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# Plant organelle targeting cell penetrating peptides

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**PLANT ORGANELLE TARGETING CELL PENETRATING PEPTIDES**

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A Thesis

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in Partial Fulfillment of the  
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**DOCTOR OF PHILOSOPHY, BIOMOLECULAR SCIENCES**

Department of Biological Sciences  
University of Lethbridge  
LETHBRIDGE, ALBERTA, CANADA

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Dedicated in loving memory of my father, Ronald MacMillan and to my mother Diane Sabourin and sisters Adrianna MacMillan and Veronica MacMillan, for their love, and support throughout my life.

## **ABSTRACT**

To address the limitations of conventional nuclear transformation I have developed a peptide based gene delivery system to genetically manipulate the genomes of plant cell mitochondria and plastids. Plant organelle targeting cell penetrating peptides (POTCPPs) are peptides that form nanoparticles composed of short peptides and double-stranded DNA (dsDNA). The peptides have cell penetrating properties and specific organelle targeting properties. These properties enabled the peptides to translocate their cargo across the cell wall and outer plasma membrane of tissue cultured plant cells to deliver dsDNA to the mitochondria or the chloroplast and have it expressed *in organello*. The POTCPP transfection method represents the first report of a successful *in vivo* plant cell mitochondrial transfection and a peptide based plastid transfection within a monocot species. The POTCPP transfection method can be applied to organelle gene targeting, functional genomic and to the study of transient gene expression in tissue cultured plant cells.

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## **LIST OF ABBREVIATIONS**

aa - amino acids

*aadA* - spectinomycin / streptomycin resistance gene

BMV- Brome mosaic virus

CD-HIT- Cluster Database at High Identity with Tolerance

CPNT - capsid protein N-terminus

CPPs - cell penetrating peptides

CPW - cell protoplast washing

cTP - chloroplast transit peptide

DNA - deoxyribonucleic acid

dsDNA - double stranded DNA

dsRNA - double stranded RNA

EF1 $\alpha$  - elongation factor1 alpha

FITC - fluorescein isothiocyanate

Fmoc - fluorenylmethoxycarbonyl

GAG - glycosaminoglycan

GFP - green fluorescent protein

GM - genetically modified

GUS -  $\beta$ -glucuronidase

HIV-1 Tat - Human Immunodeficiency Virus Type 1 Transactivator of Transcription

HR - homologous recombination

Hsp70 - heat shock protein 70

IAA - indole acetic acid

IAA-Asp - indole-3-acetyl-L-aspartic acid hydrolase enzyme

IMM - inner mitochondrial membrane

MPP - mitochondrial penetrating peptide

MS - Murashige and Skoog

mTP - mitochondrial targeting peptide

NCBI - National Center for Biotechnology Information

NLS - nuclear localization signal

NLS-PNA - nuclear localization signal peptide nucleic acids

NPC - nuclear pore complexes  
NPTII - neomycine phosphotransferase II  
PCR - polymerase chain reaction  
PEG - polyethylene glycol  
PNA - peptide nucleic acid  
*p*VEC - peptide vascular endothelial-cadherin  
qRT-PCR - Quantitative real-time PCR  
R8, R9 and R12 - oligo-arginine  
RanGAP - Ran GTPase activating protein  
RanGEF - Ran guanine nucleotide exchange factor  
RecA - recombination protein A  
RFP - red fluorescent protein  
ROS - reactive oxygen species  
RNA - ribonucleic acid  
RT - reverse transcription  
RT-PCR - reverse transcription PCR  
Rubisco - ribulose-1,5-bisphosphate carboxylase oxygenase  
SPP - stromal processing peptidase  
SS - Szeto-Schiller  
ssT-DNA - single stranded transfer DNA  
Tat - Transactivator of Transcription  
Tat<sub>2</sub> - dimer of Tat  
TIC - translocon inner chloroplast  
TIM - translocase inner mitochondrial  
TOC - translocon outer chloroplast  
TOM - translocase outer mitochondrial  
TP - transit peptide  
VirD2 - virulence protein D2  
wt - wild type

## **1.0 INTRODUCTION**

The global population is growing at an unprecedented rate; in 2010 the global population reached 7 billion people (United Nations, 2013). As a result of the ever increasing population, farmers are expected to produce increasing quantities of agricultural commodities such as food, fiber and fuel under more stringent environmental constraints. It is estimated that global crop demand will have to increase 100 – 110% by 2050 to meet future crop demands (Tilman et al., 2011). To achieve higher crop yields agricultural intensification has predominately relied upon irrigation, pesticide and fertilizer application in developed countries and land expansion in undeveloped countries (Spiertz, 2013). It is paramount that agricultural research and development focus on creating new technologies that can enhance crop yield to ensure global food security. One strategy to achieve these advances in agriculture is to focus on the development and adoption of technologies offered by the field of plant biotechnology to enhance crop improvement efforts (Moose and Mumm, 2008).

## **1.1 PLANT TRANSFORMATION BENEFITS AND LIMITATIONS**

Agricultural biotechnology currently employs plant genetic engineering methods and gene transfer technologies such as polyethylene glycol (PEG), electroporation (Díaz, 1994), microinjection (Holm et al., 2000), particle bombardment (Klein et al., 1987) *Agrobacterium* mediated gene delivery (He et al., 2010) and most recently cell penetrating peptides (Chugh et al., 2009) to introduce genetic material into plant cells. The two most prominent methods of nuclear transformation in plants are *Agrobacterium* mediated gene delivery and particle bombardment. These plant transformation methods have variable transformation rates which rely upon random integration of foreign genes

into the nuclear genome of plant cells. These random genetic insertion events often lead to positional effects on transgene expression or epigenetic gene silencing due to the production of double stranded RNA through the transcription of multiple copies or aberrant versions of the transgene (Day et al., 2000). As a result of the limitations of conventional plant nuclear transformation, novel plant transformation technologies are being developed to genetically manipulate the mitochondrial genome, which has yet to be achieved in higher plants and the plastid genome, which has only become routine for tobacco (Maliga, 2004).

Due to the hypothesized prokaryotic origin of the organelles, their genomes maintain prokaryote characteristics that facilitate organelle genetic manipulation which presents several advantages over conventional nuclear transgenic plant production (Gray, 1999). The position of genomic integration of the transgene can be controlled through homologous recombination (HR) (Verma and Daniell, 2007). Homologous recombination being the predominant mechanism utilized by the plastids and the mitochondria to integrate exogenous genetic information into the organelle genomes indicates that their recombination and repair pathways are similar (Lonsdale et al., 1988; Maréchal and Brisson, 2010). This is supported by the fact that mitochondrial and plastid recombination and repair proteins share homology as well as putative functions related to the maintenance and preservation of organelle DNA through replication, recombination and repair (Boesch et al., 2011). Targeted gene integration through HR reduces the chance of disrupting resident genes or experiencing variability in expression levels due to positional effects. The structure of the organelle genomes have retained prokaryotic gene organization as their genes are arranged into operons from which they are able to express these genes as polycistronic mRNAs (Quesada-Vargas et al., 2005). The ability to express

multiple genes in operons can be utilized to introduce entire biosynthetic pathways not previously present in the plant (Bock, 2013). High levels of transgene expression can be achieved due to cells possessing large numbers of each organelle that contain multiple organelle genome copies into which the transgene can integrate and be expressed from. Organelle number and genome copy number can vary tremendously between species, cell type and environmental growth conditions. For example beets cells can contain approximately 100 copies of the plastid genome in 19 plastids per cell compared to wheat which can have 50 000 copies of the plastid genome in 56 plastids per cell (Bendich, 1987). Plastid protein expression levels have been observed to be > 70% of total soluble protein in tobacco and lettuce (Oey et al., 2009; Ruhlman et al., 2010).

The first step in achieving high levels of protein expression is mRNA transcription. The organelles prokaryote type transcriptional components facilitate high levels of mRNA expression (Campo, 2009). RNA transcription within the organelle genome is controlled by sigma factors in plastids and mitochondria (Bohne et al., 2007). These factors are well characterized in plastids but less so in the mitochondria which may also include transcription factors. The prokaryote type transcription factors bind to prokaryote type promoters and 5' UTRs, which control phage type RNA polymerases that transcribe the organelle genes into mRNA (Maliga, 2003). Phage type RNA polymerases transcribed from the nuclear genome are translated into proteins in the cytosol and then imported into plastids and mitochondria. Plastid also encodes a supplementary polymerase within its genome whereas mitochondria do not (Liere et al., 2011). The mRNA transcribed from the organelle genome is then post transcriptionally altered through processing, splicing and editing while mRNA stability ultimately determines how



much protein is translated by the ribosomes from the mature mRNA (Germain et al., 2013).

In contrast to nuclear transformation no epigenetic instability of transgene expression in plastids has been reported (Verma et al., 2008). This eliminates the complications of gene silencing due to the production of double stranded RNA through the transcription of multiple copies or aberrant versions of transgenes that randomly integrate into the plants nuclear genome using conventional nuclear transformation technologies (Martínez de Alba et al., 2013).

Eighty percent of higher plant species including the monocotyledonous species rice, wheat and maize exhibit maternal transmission of the organelles (mitochondria and plastids) to future progeny. The maternally inherited trait of cytoplasmic male sterility impairs the production of viable pollen due to the occurrence of mitochondrial genome mutations. This trait has been applied as a strategy to control pollen production in crop breeding and hybrid maintenance (Frei et al., 2010). The limited transmission of mitochondria and plastid genetic information to pollen greatly reduces the risk of spreading genes from genetically modified (GM) crops to surrounding non-GM species through pollen flow.

Particle bombardment alternatively referred to as biolistics is the primary gene delivery technology used in both mitochondria and plastid transformation.

*Chlamydomonas reinhardtii* and two yeast species, *Saccharomyces cerevisiae* and *Candida glabrata* mitochondria have been transformed using the biolistic method (Remacle et al., 2006; Zhou et al., 2010). It has also been successfully used to achieve stable plastid transformation in dicot species such as tobacco (Svab and Maliga, 1993), *Arabidopsis* (Sikdar et al., 1998), potato (Sidorov et al., 1999), tomato (Ruf et al., 2001),

*Lesquerella fendleri* (Skarjinskaia et al., 2003), oilseed rape (Hou et al., 2003), soybean (Dufourmantel et al., 2004), petunia (Zubko et al., 2004), cotton (Kumar et al., 2004), carrot (Kumar et al., 2004), poplar (Okumura et al., 2006), cauliflower (Nugent et al., 2006), lettuce (Kanamoto et al., 2006), cabbage (Liu et al., 2007), sugar beet (Marchis et al., 2009) and alfalfa (Wei et al., 2011). In contrast only a limited number of monocot species such as rice, wheat and recently sugarcane produced preliminary plastid transformation results of which wheat and sugarcane have yet to be verified (Lee et al., 2006; Cui et al., 2011; Khan, 2012). The lack of transferability of dicot plastid transformation methods to monocot plastid transformation indicates that a species barrier exists.

Similar challenges were experienced while developing *Agrobacterium* mediated gene delivery methods in monocots. Initially the use of *Agrobacterium tumefaciens* as a transformation system was limited to dicotyledonous plant species (Horsch, 1985). The reason why *Agrobacterium*'s host range was perceived to be restricted to dicotyledonous plant species was unclear, as an extensive review of plant species susceptibility to *Agrobacterium* infection was performed at University of Ghent, Belgium, in 1976. This study included some monocotyledonous species (De Cleene and De Ley, 1976). It is now known that *Agrobacterium* spp., have an extremely broad host range that includes dicots, monocots, gymnosperms, fungi and even human cells (Kunik et al., 2001; Lacroix et al., 2006). This fact, along with the advancements made in plant biology, tissue culture and molecular biology have led to the expansion of *Agrobacterium* plant transformation into recalcitrant plant species and explains why *Agrobacterium* mediated plant transformation is one of the world's leading nuclear plant genetic engineering technologies (Anami et al., 2013).

## 1.2 CELL PENETRATING PEPTIDES

To overcome the previously stated limitations of conventional nuclear, mitochondrial and plastid transformation technologies, investigations into the use of peptide based genetic transformation methods, developed to manipulate mammalian cells, have been applied to plants (Eudes and Chugh, 2008). Unfortunately, plants are recalcitrant to most of these methods. Peptide transfection technology such as cell penetrating peptides (CPPs) is the exception and is currently emerging as a viable plant transfection technology (Chugh et al., 2010).

Cell penetrating peptides are defined as short cationic and or amphiphathic peptides which are capable of mediating the intracellular delivery of polar hydrophilic compounds such as proteins or oligonucleotides by transducing their polymeric cargo across cell membranes in a receptor independent manner (Veldhoen, 2008). Cell penetrating peptides generally have a large positive charge due to the high concentration of basic amino acid residues (arginine and lysine) contained within the peptide sequence. Arginine and lysine residues are strongly attracted to the negatively charged plasma membrane of cells. The plasma membrane contains a large number of anionic compounds: phosphate groups in the phospholipids and glycosaminoglycans (GAGs) that extend from the surface of the plasma membrane. The electrostatic attraction between the cationic peptide and the anionic groups of the plasma membrane result in aggregation of the peptide at the cell surface (Vives, 2003). As the guanidinium groups interact with the anionic compounds, they become less polar through charge neutralization which alters the adaptive solubility of the peptide allowing it to enter and pass through the plasma membrane (Pantos et al., 2008).

One major concern while utilizing CPPs to deliver cargo into cells is cytotoxicity. Depending on the type and concentration of CPP it has been demonstrated that CPPs disturb intracellular  $\text{Ca}^{2+}$  concentrations during membrane translocation into Jurkat and HeLa cells (Lorents et al., 2012). Once a threshold level of cytosolic  $\text{Ca}^{2+}$  is reached membrane repair mechanisms are triggered to counteract the disturbances caused by peptide internalization. Without such mechanisms cells are killed. Although the cellular mechanisms weren't identified similar cytotoxic effects have been observed while using CPPs to transfect dsDNA into plant cells (Shim et al., 2013). Both of these examples indicate that to effectively utilise CPPs to deliver cargo into cells, peptide concentration has to be minimized.

### **1.3 MECHANISMS OF INTERNALIZATION**

Internalization or membrane translocation of CPP cargo complexes can follow one of two pathways: transient permeabilization or an endocytotic pathway. Direct cell penetration involves, inverted micelle and aqueous toroidal pore formation which are passive, energy independent routes of entry across the hydrophobic core of cell membranes (Guy and Li, 1998; Gould et al., 2008; Herce and Garcia, 2008).

There is a considerable amount of variability among the endocytic pathways utilized during CPP cargo complex internalization. These pathways have evolved to segregate the intracellular environment from the extracellular environment. They are energy dependent and are characterised by the formation of vesicles to internalize fluids, solutes and macromolecules. CPPs can be internalized through at least four major endocytotic pathways; macropinocytosis, clathrin mediated, caveolin mediated, and clathrin and caveolin independent endocytosis (Patel et al., 2007).

Macropinocytosis is involved with immune surveillance by directly sampling large volumes of the extracellular fluid. Bacterial pathogens exploit this mechanism of entry by injecting toxins into the membrane. This was shown to stimulate membrane ruffling thereby inducing Rho-family GTPases to activate the actin-dependent formation of large endocytic vesicles. These vesicles ranging in size from 1 – 5  $\mu\text{m}$  (Conner and Schmid, 2003). Macropinocytosis has been proposed as a predominant mechanism of CPP internalization in live cells (Kaplan et al., 2005).

Clathrin mediated endocytosis is the best characterized of all the endocytic pathways. Clathrin coated vesicles form on the outer plasma membrane surface. Clathrin forms a lattice structure that deforms the membrane into a curved bud. These clathrin coated pits contain adaptor proteins that bind to the cytoplasmic domains of transmembrane molecules. They selectively sequester proteins in a receptor mediated fashion. Receptor - ligand interaction activates the internalization of clathrin coated vesicle. The clathrin coat is shed once it is internalised. The uncoated endocytic vesicle can develop into an early endosome which matures into a late endosome through acidification by lysosomes or develops into a protein recycling endosome that returns receptors back to the plasma membrane (Brodsky et al., 2001). Clathrin coated vesicles are approximately 120 nm in size (Conner and Schmid, 2003). Fluorescently labelled Tat peptide has been described as being directed into the clathrin-mediated endocytic pathway as a method of internalization (Richard et al., 2005).

Caveolae are involved with endocytosis, transcytosis, and various signalling pathways. Different catalysts: sterols, glycosphingolipids and pathogens such as the Simian Virus 40 (SV40) have been shown to stimulate caveolae internalisation in mammalian cells. Caveolae are flask shaped invaginations that form at the plasma

membrane surface within areas called lipid rafts. These areas of the plasma membrane contain high concentrations of sphingolipids, and cholesterol. The structure and shape of caveolae form due to the polymerisation of the dimeric caveolin protein that binds to cholesterol in the plasma membrane. Caveolae are slowly internalised and form detergent resistant vesicles between 60 – 80 nm in diameter (Parton and Simons, 2007). It has been demonstrated that caveolar endocytosis is initiated in cell membrane lipid rafts and involved with Tat fusion protein internalization (Fittipaldi and Giacca, 2005).

Other less defined endocytic pathways such as clathrin and caveolin independent endocytosis, or lipid raft endocytosis are involved with CPP internalization as well. The type of endocytotic pathway involved in CPP internalization will determine the outcome of the CPP cargo complex. Vesicle contents maybe degraded in lysosomes, be stored in caveosomes, or transported to the endoplasmic reticulum or golgi complex in route to their final target. CPPs must therefore escape the vesicles to reach their intended target before they are sorted to the cellular compartment specified by the type of endocytic pathway involved.

#### **1.4 ENDOSOMAL ESCAPE**

Endosomal escape is a limiting factor in determining if a CPP reaches its final target. Endosomal disruptors, such as chloroquine and polyethylenimine are thought to facilitate membrane rupture and escape from endosomes though their ability to buffer the endosomal compartment (Liang and Lam, 2012). Fusogenic peptides such as the N-terminal 20 amino acid peptide derived from the influenza virus hemagglutinin protein (HA2), destabilizes lipid membranes at low pH to increase endosomal escape and enhance CPP cargo delivery (Han et al., 2001). Once gaining access to the cytoplasm

CPPs can localize to various subcellular organelles within the cell. This review will primarily focus on information related to cell penetrating peptides and their application to the transfection of nucleic acids to the nucleus, mitochondria and in the case of plant cells the plastid.

## **1.5 NUCLEAR PEPTIDE TRANSDUCTION**

The nucleus is one of the defining intracellular organelles common to all eukaryotic cells. It contains genetic information encoded in DNA molecules that are organized into genes and stored as chromosomes. Genes within the chromosome are transcribed into mRNA and then exported to the cytosol to be translated into proteins by ribosomes. The fate of these nascent proteins is determined by the subcellular localization signal encoded by the proteins amino acid sequence. The subcellular localization signal directs the delivery of the protein to specific locations or organelles such as the nucleus, mitochondria or chloroplast within cells.

The nucleus is a membrane bound organelle with an average diameter of 11 to 22  $\mu\text{m}$  depending on the cell type. Macromolecules are actively transported across the nuclear membrane through nuclear pore complexes (NPC). Molecules with a diameter  $< 9$  nm or 60 kDa can passively diffuse through the NPC. The NPC is capable of dilating 10 - 25 nm in diameter to facilitate the active transport of large molecules. Directionality of nuclear transport is maintained by Ran GTPases acting as a molecular switch controlling the binding and release of cargo through the control of Ran guanine nucleotide exchange factor (RanGEF) generating Ran-GTP in the nucleus and Ran GTPase activating protein (RanGAP) hydrolysing Ran-GTP to Ran-GDP in the cytosol (Lechardeur and Lukacs, 2006).

Nuclear proteins require a nuclear localization signal (NLS) to be considered as a target for the nuclear import pathway mediated by the importin  $\alpha/\beta$  also called karyopherin proteins (Inoue et al., 2005). Importin  $\alpha$  has two NLS binding sites where it can bind proteins containing a classical NLS or a bipartite NLS. Classical or monopartite NLSs are composed of a single N-terminal region of successive basic amino acids whereas the bipartite NLS is composed of 2 regions of successive basic amino acids separated by a linker region of 10 – 12 amino acids (Lange et al., 2007). Binding of a NLS varies due to differences in affinity to the various sub types of importins found in different species (Kosugi et al., 2009). Nuclear localization signals can bind to importin  $\alpha$ , importin  $\beta$  or importin  $\alpha/\beta$ ; the duplex or trimeric complex docks with the NPC. Translocation through the NPC occurs through an uncharacterized mechanism. Upon entering the nucleoplasm Ran in its GTP bound form Ran-GTP binds to importin  $\beta$ . This causes the dissociation of the trimeric complex, releasing importin  $\alpha$  and the cargo. The importins are translocated back to the cytoplasm where Ran-GTP is hydrolysed to Ran-GDP ready to complete another nuclear import cycle.

Many NLSs have been identified and applied to the study of directed nuclear import because protein and nucleic acid transport into the nucleus is otherwise a rare and inefficient event due to the impermeable nature of the nuclear membrane. The best characterized classical or monopartite NLS sequence is the SV40 large T-antigen NLS 126-132 (PKKKRKV). The peptides nuclear import is dependent on SV40 binding to importin  $\alpha$  (Harreman et al., 2004). Binding of SV40 to plasmid DNA through covalent, non-covalent or hybridization to NLS peptide nucleic acids (NLS-PNA) has been demonstrated to increase nuclear import (Cartier and Reszka, 2002). Transfection rates in these experiments increased over control values using SV40 but at low levels due to a



lack of cellular permeability. This was overcome by developing chimeric peptides through the conjugation of NLSs to CPPs or the identification of NLSs with CPP properties.

Chimeric peptides such as MPG is composed of the lysine-rich domain derived from the hydrophilic domain of the NLS from SV40 large T antigen (KKKRKV), and the N-terminal hydrophobic domain from the HIV protein gp41 (GALFLGFLGAAGSTMGA) which are separated by a flexible linker domain. This amphipathic peptide is capable of complexing DNA in a non-covalent manner and effectively transporting it into mammalian cells (Morris et al., 1997). An example of a protein that contains a NLS and was identified as having intrinsic cellular transduction properties was the Human Immunodeficiency Virus Type 1 Transactivator of Transcription (HIV-1 Tat).

Characterization of HIV-1 Tat led to the identification of an amino terminal stretch of basic amino acid residues 49–57 (RKKRRQRRR) within the Tat protein that confers both cell penetrating and nuclear localization properties to the protein (Vives et al., 1997). The essential elements responsible for Tat's ability to cross plasma membranes are the guanidinium head group of the arginine residues and the resulting peptide's high net positive charge. In spite of the progress made in characterizing CPP transduction mechanisms much debate still exists within the scientific community regarding the mechanisms of CPP plasma membrane transduction.

The majority of CPP transfection studies have occurred in mammalian cells but the gradual accumulation of plant based CPP experiments has steadily increased over the last 10 years. Initial plant based CPP studies focused on the transduction of a synthetic peptide, histone proteins (H2A and H4), transportan, TP10, penetratin and peptide

vascular endothelial-cadherin (*pVEC*) into dicot plant tissues (Cormeau et al., 2002; Rosenbluh et al., 2004; Mäe et al., 2005). This preliminary work established that CPPs could enter plant cells setting up the next logical extension - the testing of peptide based transduction of macromolecular cargo into plant cells. It was determined that double stranded RNA (dsRNA) could be delivered in a non-covalent manner using R12 to silence  $\beta$ -glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) in transgenic tobacco cells (Unnamalai et al., 2004). This was the first example of protein transduction technology being utilized to deliver nucleic acids to induce post-transcriptional gene silencing in plant cells. Next it was demonstrated that green fluorescent protein (GFP) or red fluorescent protein (RFP) covalently linked to Tat or R9 as well as in a non-covalent manner in a later experiment, could transduce these proteins into tomato root tip cells, onion epidermal and root tip cells as well as corn root tip cells (Chang et al., 2005; Wang et al., 2006; Chang et al., 2007). A subsequent variation of these experiments was to utilize Tat and R9 to simultaneously deliver GFP and RFP into onion root tip cells (Lu et al., 2010). The experimental evidence that CPPs could transduce cargo into plant cells was applied to the covalent and non-covalent delivery of indole-3-acetyl-L-aspartic acid hydrolase enzyme (IAA-Asp) using Tat to inhibit mung bean seed germination (Liu et al., 2007). Their study revealed that the covalently linked Tat-enzyme complex inhibited seed germination and root development more than the non-covalent complex due to higher indole acetic acid (IAA) levels measured in the treated mung bean seeds when compared to the control. These indirect findings would have benefited from direct protein analysis methods such as western blots to quantify transduced IAA-Asp enzyme levels in the seeds post-delivery to determine if these results correlate with germination rates and measured IAA levels. These preliminary experiments established that CPPs could

transduce individually or in conjunction to cargo bound either covalently or non-covalently into plant cells establishing the utility of CPPs in plant sciences.

Recognising the agricultural biotechnological value of protein transduction technology, Tat, a dimer of Tat (Tat<sub>2</sub>), pVEC and transportan were tested in the monocot species triticales (Chugh and Eudes, 2007; Chugh and Eudes, 2008). Tat, Tat<sub>2</sub>, pVEC and transportan were found to transduce across the outer plasma membrane of triticales mesophyll protoplasts and accumulate in the nucleus of these cells. Tat, Tat<sub>2</sub>, pVEC, transportan and Pep1 were also found to transduce into isolated triticales microspores (Chugh et al., 2009). Interestingly pVEC and transportan showed highly variable internalization into different triticales tissues: leaf tips, leaf base, coleoptiles and root tips. This variability in CPP internalization is congruent with what has been observed in testing CPP internalization in various cell types (Liu et al., 2008; Sugita et al., 2008). Protoplasts are plant cells that have had their cell wall enzymatically removed therefore present less of a barrier to CPP cargo transduction. In contrast intact plant tissues such as the leaf tips, leaf bases, coleoptiles, microspores and root tips are less permeable due to the presence of a cell wall surrounding these plant cells.

The unique feature of the cell wall was considered by Mizuno et al. (2009) when investigating how the various lengths and cargo position in oligo-arginine affect CPP cargo transduction into plant cells. They determined that R8 with cargo attached to the C-terminus as opposed to the N-terminus of the peptide resulted in the highest rate of cellular transduction in tobacco cell suspension culture (Mizuno et al., 2009). Plant cell wall composition changes as plant cells mature. A point that could be considered in future CPP plant cell suspension experimentation is the timing of transfection. Analysis of *Arabidopsis* cells grown in culture were shown to have their cell walls increase in

thickness and average stiffness during exponential growth phase in comparison to stationary phase (Radotić et al., 2012). This study reinforces the concept that each CPP requires individual optimization for the cell type and cargo being transduced to achieve maximum cargo delivery in that particular system (Keller et al., 2013).

Efforts to enhance plant cell protein transduction across the cell wall and into somatic plant cell tissues have involved the application of cell permeabilization agents. Chugh et al. (2008) demonstrated that wheat immature embryos treated with a toluene-ethanol solution increased cellular uptake of Tat, M-Tat, Tat<sub>2</sub>, *pVEC* and transportan versus non-treated embryos. In addition it was also shown that Pep1, Tat and Tat<sub>2</sub> could non-covalently transduce the GUS protein as well as plasmid DNA encoding the *uidA* gene into permeabilized wheat embryos or isolated microspores (Chugh et al., 2009). The addition of Lipofectamine<sup>®</sup> (Invitrogen<sup>™</sup>) further enhanced Tat<sub>2</sub> *uidA* gene delivery and expression in embryos and microspores (Chugh and Eudes, 2008).

Recent developments in plant based nuclear protein transduction technology have been in the identification of CPPs related to plant pathogenesis. A 22 amino acid N-terminal peptide from the capsid protein (CPNT) derived from the monocot infecting brome mosaic virus (BMV) was found to translocate into barley and *Arabidopsis* mesophyll protoplasts and root tip and hair cells. The CPNT CPP was also utilized to deliver protein (GFP) in a covalent and non-covalent manner as well as nucleic acids (dsRNA) in a non-covalent fashion (Qi et al., 2010). Another recently discovered CPP Bp100 is a synthetic peptide based on a mutated version of the naturally occurring antimicrobial peptide cecropin-A. Bp100 was found to internalize independently and when covalently bound to the actin binding protein Lifeact in tobacco cell suspension culture (Eggenberger et al., 2011).

All of these studies illustrate that many variable factors such as peptide concentration, amino acid sequence, peptide structure, cargo attachment, cell type and a variable unique to plants, cell wall influence CPP transduction (Milletti, 2012). All of these factors affect the cellular transduction mechanisms utilized by CPPs to enter cells and the peptides final subcellular localization. Two other plant cell organelles (mitochondria and plastids) containing DNA, are important to plant primary metabolism. These organelles could be viable targets for genetic manipulation using peptide transduction technology.

## **1.6 MITOCHONDRIAL PEPTIDE TRANSDUCTION**

Currently mitochondrial research in both plant and animal cells is limited due to the inability to manipulate the mitochondria genome in whole cells. The mitochondria plays a central role in eukaryotic metabolism but due to an inability to genetically modify the mitochondrial genome, many aspects of its involvement in metabolism, cellular physiology, and general cell biology have yet to be fully defined.

Mitochondria are surrounded by a double membrane and have an average diameter of 0.5 to 1  $\mu\text{m}$  depending on the cell type. The outer and inner membranes are used to generate an electrochemical proton gradient to produce ATP through the electron transport chain. Plant mitochondrial genomes vary considerably from the smallest in the alga species *Polytomella capuana* a linear fragment of 13 kb, to the largest in zucchini species *Cucurbita pepo* 983 kb (Smith and Lee, 2008; Alverson et al., 2010).

Mitochondrial genomes were greatly reduced through gene loss and transfer to the host cell nucleus during the co-evolution of the mitochondrial in the eukaryotic cell (Adams and Palmer, 2003). Now, most of the required mitochondrial proteins are transcribed from

the cells nuclear genome. Mitochondrial proteins transcribed from the nucleus are synthesized on polyribosomes in the cytosol.

Precursor proteins contain N-terminal mitochondrial targeting peptide (mTP) sequences which are between 20-80 amino acids long. They form amphiphilic  $\alpha$ -helices that are composed of basic amino acids. Alanine and serine are also highly represented within mTP sequences.

As the precursor protein is synthesized, cytosolic factors such as Hsp70, mitochondrial import stimulation factor and presequence binding factor bind to the precursor protein and facilitate its import into the mitochondrial matrix (Zhang and Glaser, 2002). Cytosolic precursor proteins are transported across the double membrane by mitochondrial translocases of the outer and inner membranes (TOM and TIM). Once a protein is imported into the matrix of the mitochondria the mTP sequence is cleaved off the protein by a mitochondrial processing peptidase (Zhang and Glaser, 2002).

The use of mTPs in protein transduction technologies has largely been restricted to gene modification and gene delivery therapeutic strategies as treatments to mitochondrial diseases in mammalian cells. The import of a mTP conjugated to a peptide nucleic acid (PNA) was applied as a strategy to selectively inhibit the replication of mutant mtDNA in human cells (Chinnery et al., 1999). Only a limited amount of success was achieved using this method due to the lack of inner mitochondrial membrane (IMM) penetration of mTP-PNAs. It was also reported that oligonucleotides conjugated to mTPs could be imported into the mitochondrial matrix of isolated mitochondria (Flierl et al., 2003). This approach to mitochondrial gene therapy is currently limited by the size of the DNA that can be delivered to the matrix.

Numerous peptide constructs exhibiting mitochondrial targeting properties have been validated as viable mammalian mitochondrial delivery strategies. A series of synthetic mitochondrial targeted antioxidant peptides were developed to reduce ROS and cell death caused by mitochondrial dysfunction (Zhao et al., 2004). Szeto-Schiller (SS) peptide antioxidants were developed as tetrapeptides with a structural motif of alternating aromatic residues containing antioxidant properties and basic residues with mitochondrial cell penetrating properties (Szeto, 2006). The net positive charge of these peptides facilitates the charge driven accumulation within the inner mitochondrial membrane (IMM). A similar strategy was used to develop mitochondrial penetrating peptides (MPP) (Horton et al., 2008). These short synthetic peptides are made up of alternating cationic residues which contribute to the peptides net positive charge and hydrophobic residues which provide the peptides with their lipophilic characteristics. Mitochondrial penetrating peptides gain access to the IMM due to their combined cationic and lipophilic nature. These peptides were conjugated to various bioactive molecules for their delivery into the mitochondria (Yousif et al., 2009). Understanding the physicochemical properties responsible for mitochondrial targeting and applying a systematic approach to the development of new peptides may prove to be an effective strategy in targeting therapeutics and nucleic acids to the mitochondria of both plant and animal cells in the future.

## **1.7 PLASTID PEPTIDE TRANSDUCTION**

Plastids are generally present in most plant cells, with chloroplasts being one of the most well studied. Chloroplasts contain chlorophyll, a green pigment involved in photosynthesis or light driven ATP synthesis. Just like the mitochondria, the chloroplast

is an organelle made up of a double membrane structure. Unlike the mitochondria, chloroplasts contain a third inner membrane called the thylakoid contained within the stroma; the central space of the chloroplast. The thylakoid membrane contains all the components of the photosynthetic electron transfer system used to sequester energy during photosynthesis. Chloroplasts have a diameter of between 2 to 10  $\mu\text{m}$  and contain multiple copies (1000 – 10 000) of the plastid genome (Shaver et al., 2006; Zoschke et al., 2007). Higher plant plastid genomes range in size from 120 to 218 kb (Wakasugi et al., 2001; Chumley et al., 2006). The majority of plastid proteins are encoded in the nuclear genome. Like mitochondrial proteins these nuclear encoded plastid proteins are synthesized on polyribosomes in the cytosol. These cytosolic precursor proteins contain N-terminal chloroplast transit peptide (cTP) sequences that interact with cytosolic factors that facilitate the targeting of precursor proteins to plastids (Li and Teng, 2013). The precursor protein is transported across the double membrane by the chloroplast outer and inner membrane translocons (TOC and TIC) (Inoue and Keegstra, 2003). Once the protein is imported into the stroma of the chloroplast the cTP sequence is cleaved off of the protein by a stromal processing peptidase (SPP).

The primary application of transit peptide sequences has been in their use to characterize plastid protein targeting from nuclear expressed genes. Transit peptide sequences differ in terms of length 13 – 146 amino acids and in amino acid composition (Kessler et al., 1994). Transit peptides can adopt a  $\alpha$ -helical structure, contain short motifs but generally lack consensus which complicates TP prediction (Bruce, 2001; Li and Chiu, 2010). Recently, a multi-selection and multi-order model was proposed to explain the lack of sequence identity in TP sequences (Li and Teng, 2013). In presenting their idea they compare transit peptides to gene promoters. In that, TPs are made up of TOC/TIC



binding motifs that can be arranged in various configurations just like the *cis* elements contained in promoter elements. The interchangeability of these binding motifs within TP sequences allows plastids to regulate precursor protein translocation (Li and Teng, 2013). This type of regulatory mechanism would allow plastids to titrate the concentration of nuclear encoded plastid proteins as well as target these proteins to different types of plastids at different maturity levels. If this hypothesis is correct, it would address the long standing enigma of TP variability. This new concept could be developed into a biotechnology platform to target proteins or nucleic acids not just into the chloroplast but to all the various plastid types that exist in different plant tissues.

The allotropic expression of proteins refers to the nuclear expression of a gene that normally resides in an organelle genome. This strategy was applied to investigate enzyme assembly and functionality of the large catalytic subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco) in the tobacco plastid (Kanevski and Maliga, 1994). The *rbcL* gene was deleted from the plastid genome, then modified to contain an N-terminal TP sequence from the pea rubisco small subunit. The modified *rbcL* gene was inserted into the nuclear genome. Expression of the modified *rbcL* protein demonstrated that the protein could be successfully targeted to the chloroplast where it assembled into a functional enzyme and complemented the mutated plastid genome.

Transit peptide sequences were also utilized by Primavesi et al., (2008) to target the GFP protein to visualize non-green plastids in different tissues of wheat. The TP sequences from rice FtsZ, maize ferredoxin III and the maize rubisco small subunit were linked to GFP. All of the nuclear encoded fusion proteins were targeted to non-green plastids. This allowed them to visualize photosynthetically inactive plastids in wheat endosperm, pollen and roots tissues (Primavesi et al., 2008). All of these examples

demonstrate that TP sequence can be utilized to target and deliver proteins into monocot plastids.

The investigation of novel plastid transformation technologies is essential to achieving stable monocot plastid transformation methods. Existing plastid transformation technologies like particle bombardment have not performed well in monocots. If monocotyledonous plastid transformation is to be developed into a mature technology the application of standard plant tissue culture and selection methods needs to be examined while the exploration of new alternative organelle transformation technologies such as peptides are pursued.

## **1.8 FUTURE DIRECTIONS OF PEPTIDE TRANSDUCTION**

Recent efforts to overcome the recalcitrance of monocots to classical plant transformation technologies such as *Agrobacterium* mediated transformation or particle bombardment have necessitated the use of nanoparticle complexes composed of peptides, proteins and cationic lipids to facilitate plant transformation. Ziemienowicz et al., (2012) used a novel approach that tested the ability of virulence protein D2 (VirD2), recombination protein A (RecA) bound to single stranded transfer DNA (ssT-DNA) and various combinations of Tat<sub>2</sub> and the cationic lipid Lipofectamine to deliver VirD2-ssT-DNA-RecA nanocomplexes into triticale microspores. VirD2 is one of the virulence proteins used by *Agrobacterium* to guide and integrate the bacterium's infecting ssT-DNA into the host plants genome (Gelvin, 2000). RecA is a recombination and repair protein involved in DNA strand exchange, renaturation and DNA dependent ATPase activities (Otero and Hsieh, 1995). It was previously shown by Ziemienowicz, et al. (2001) that RecA could substitute for the *Agrobacterium* VirE2 protein during nuclear

import of T-DNA (Ziemienowicz et al., 2001). The novelty of this approach excludes the bacterium and focuses on the *in vitro* assembly of Tat<sub>2</sub> and Lipofectamine to enable the transduction of the VirD2-ssT-DNA-RecA nanocomplex into the microspore while preventing nucleolytic degradation of the DNA, followed by VirD2 and RecA facilitating ssT-DNA nuclear import and genomic integration. This method resulted in intact transgene incorporations into the triticale genome. Further refinement of the procedure by varying the ratio of the nanocomplex components may lead to limiting copy number and achieve single copy transgene integration in a larger proportion of the transgenic plant population. This is a significant achievement over both *Agrobacterium*-mediated and particle bombardment transformation methods which often result in aberrant transgene integration patterns.

A more recent study also tested nanoparticle complexes to transfect linear double stranded DNA (dsDNA) containing the *uidA* marker gene into triticale microspores (Shim et al., 2013). The CPPs Tat<sub>2</sub>, Pep1 and the cationic lipid Lipofectamine were combined in various concentrations to transduce dsDNA into microspores. RecA was co-delivered to determine if the repair protein would preserve the integrity of the donor dsDNA. These experiments determined that Tat<sub>2</sub> was more effective at creating transgenic plants than Pep 1. An explanation of why more transgenic plants were produced with Tat<sub>2</sub> could be due to the antimicrobial properties of Pep1. Pep1 is an antimicrobial peptide and has been shown to be effective against gram-positive and gram-negative bacteria (Zhu et al., 2006). These properties would result in reduced viability of the transfected microspores. RecA also reduced transgenic plant production when co-delivered with donor DNA in microspores. It was determined that RecA had a slight cytotoxic effect but preserves transgene integrity when inserted into the triticale genome (Ziemienowicz et al., 2012).

Again, varying the ratio of the nanocomplex components may lead to limiting copy number and achieve single copy transgene integration. This would ultimately reduce the incidence of transgene silencing in transgenic plants production.

Together, both of these studies highlight the use of peptides as effective plant macromolecular transporters and demonstrate important technical advances in monocot transgenic plant production methods. These studies also demonstrate that peptides used in plant transformation could lead to other major advancements in plant functional genomics. This is not only applicable to monocots but also dicots due to their ease of use and versatility in delivering proteins and nucleic acids as well as their ability to be used in combination with other transduction technologies. These innovative multi component protein transduction systems are universal in their application making them broad based platform technologies that will advance plant science and agricultural biotechnology. The application of CPPs to plant transformation illustrates that plant based CPP discovery and protein transduction are an increasingly attractive area of scientific research. Having the ability to directly manipulate plant organelle genomes will facilitate organelle functional genomics, the manipulation of plant traits of agronomic importance and the creation of new commercial food and industrial crop species.

## **2.0 PLANT CHLOROPLAST / PLASTID TARGETING CELL PENETRATING PEPTIDES.**

### **2.1 INTRODUCTION**

The endosymbiotic theory explains the origin of the plastid and mitochondrion organelles as a series of ancient endosymbiotic events involving an ancestral host cell and prokaryotic endosymbionts (Sagan, 1967). During the evolution of the chimeric cell; the plastid and mitochondrial genomes were greatly reduced through gene loss and transfer to the host cell nucleus. Now, most of the required chloroplast / plastid and mitochondrial proteins are transcribed from the cells nuclear genome, translated in the cytoplasm and transported into the organelles by hetero-oligomeric protein complexes.

Transport of nascent cytoplasmic proteins is directed by protein sorting signals that primarily occur at the amino terminus, but can occur at the carboxyl terminus or within the protein sequence as well (Nakai, 2000). Proteins targeted to plastids contain protein sorting signals which are distinct peptide sequences referred to as chloroplast transit peptide (cTP) sequences. They are recognized by the plastid protein translocation machinery which facilitates the transport of these nascent cytosolic proteins across the organelles' double membrane. Chloroplast transit peptides vary in length from 13 – 146 amino acids (aa), can adopt an  $\alpha$ -helical structure, contain short motifs but generally lack consensus which has complicated cTP prediction (Bruce, 2001; Li and Chiu, 2010).

An example of a protein that contains a protein sorting signal and targets the nucleus is the Human Immunodeficiency Virus Type 1 Transactivator of Transcription (HIV-1 Tat). This protein activates viral gene expression and replication. It does so by crossing the outer plasma membrane and accumulates in the nucleus of cells due to the

presence of a nuclear localization signal (NLS) within the protein sequence. This was independently demonstrated by Frankel and Green (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Subsequent characterization of HIV-1 Tat lead to the identification of an amino terminal stretch of basic amino acid residues 49–57 (RKKRRQRRR) referred to as Tat. Further experimentation confirmed Tat as being HIV-1 Tat proteins NLS and responsible for conferring cell penetrating and nuclear localization properties to the HIV-1 Tat protein. These findings lead to the discovery that Tat when conjugated to macromolecular cargo (nucleic acids or proteins) could be utilized as a molecular transport protein due to its cell penetrating properties. Tat and many other CPPs have since been identified and utilized as molecular transporters in plant cells (Zonin et al., 2011; Lakshmanan et al., 2012).

Monocotyledonous plastid transformation has received much attention but has seen very little success in being developed into a routinely used technology. The major factors obstructing the production of homoplasmic monocots is the lack of a stringent selection method and a gene delivery system compatible with a cell culture system that supports multiple rounds of selection to achieve homoplasmy. Primary plastid transformants are often composed of a combination of wild type plastid genomes and plastid genomes containing your gene of interest; this is referred to as heteroplasmy. To eliminate the existing wild type copies of the plastid genome and select for the new transgenic plastid genome containing your gene of interest, a state called homoplasmy requires multiple rounds of tissue culture while applying stringent selection (Maliga, 2004). Previous studies have produced transient plastid expression in rice (GFP) and as yet to be confirmed in wheat (GUS) (Daniell et al., 1991; Khan and Maliga, 1999). The

production of fertile heteroplasmic transplastomic rice has since been achieved but all have fallen short of achieving homoplasmy (Lee et al., 2006).

Biolistics is currently the preferred method to transform plastid genomes (Elghabi et al., 2011). This method uses high pressure helium to accelerate microcarriers to penetrate the cell wall of plant cells and deliver nucleic acids to the nucleus or plastids in plant cells. *Agrobacterium* and PEG mediated plastid transformation methods were successfully applied to plastid transformation but with only limited success (Deblock et al., 1985; Spörlein et al., 1991). These limitations have led to biolistic plastid transformations popularity and broad spectrum of application.

This study proposes a cTP plastid transfection method as an alternative strategy to the primary method of biolistics used to introducing genetic material into the plastid genome. Due to the cell penetrating and nuclear localization properties known to be possessed by Tat, it is reasonable to purport that other peptides could have similar cell penetrating and organelle targeting properties. We report the discovery of cTPs that have both these properties. These cTPs were used to translocate a linear dsDNA *aadA:gfp* reporter cassette in a non-covalent manner across the outer plasma membrane into tissue cultured protoplasts and microspores and have it expressed.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 IDENTIFICATION OF CHLOROPLAST TARGETING PEPTIDE SEQUENCES**

Available protein sequences for wheat, rice, maize, and *Arabidopsis* were downloaded from National Center for Biotechnology Information (NCBI) GenBank (09,

2008). To eliminate sequence redundancy within the protein sequence datasets, the computer software program Cluster Database at High Identity with Tolerance (CD-HIT) was used to generate a set of non-redundant sequences (Huang et al., 2010). The protein sequences were then analyzed using the TargetP software program to identify N-terminal protein sorting signal sequences specifically, chloroplast transit peptide (cTP) sequences as well as to predict the subcellular localization properties of these predicted N-terminal protein sorting signal sequences (Emanuelsson et al., 2000). TargetP uses an algorithm that incorporates a neural network to calculate a transit peptide score and weighted matrices to locate the transit peptide cleavage site. The cTP sequence dataset was parsed according to specific selection criteria to identify cTP sequences with similar physicochemical properties (lipophilic and a net positive charge due to a high concentration of basic aa) as cell penetrating peptide. To be selected as a candidate sequence the sequence had to have a TargetP relative confidence value of  $\geq 0.9$ , a sequence length of  $\leq 35$  aa and an average hydrophilicity  $\leq 0$ .

### **2.2.2 PEPTIDE SYNTHESIS AND LABELING**

Twenty three candidate cTP sequences with the highest values selected from those meeting all the criteria outlined above were synthesized using solid phase fluorenylmethoxycarbonyl (Fmoc) chemistry by CanPeptide Inc., Canada. Refer to (Table 2.1) for a full list of the synthesized peptides. Each peptide was labelled with fluorescein isothiocyanate (FITC) at the N-terminus, to facilitate visual detection by fluorescence.



**Table 2.1 Candidate chloroplast targeting cell penetrating peptides.**

The table describes peptide name, overall charge, hydrophilic value, peptide amino acid sequence and the species from which it was derived.

cIP	Peptide Charge	Hydro-philicity	# aa	Target P Confidence Level	Peptide sequence	Species	GenInfo Identifier
cIP1	2.9	0	17	0.96	MGGCVSTPKSCVGAQLR	<i>Arabidopsis</i>	GI:5091532
cIP2	4.2	0	31	0.94	MQTLTASSVSSIQRHRPHAGRRSSSVTF	Wheat	GI:112181147
cIP3	2.0	-0.1	15	0.94	MKNPPSSFASGFGR	Rice	GI:34394426
cIP4	2.1	-0.2	32	0.96	MAALIPAIASLPRAQVEKPHMPVSTRPGLVS	Wheat	GI:111055216
cIP5	2.0	0	33	0.99	MSSPPPLFTSCLP ASSPSIRRDSTSGSVTSPLR	<i>Arabidopsis</i>	GI:125537923
cIP6	5.1	0	34	0.95	MAATTSTTPAVSFLSPSPHRQGAPRRRAPFLLR	Maize	GI:195656083
cIP7	4.2	-0.2	33	0.95	MPVPHLLSPLPLSPPPPLHRNGAPWRRPTQLR	Maize	GI:195642206
cIP8	5.0	-0.2	35	0.97	MALKFNPLVASQPYKFPSSTRPPTPSFRSPKFLCL	<i>Arabidopsis</i>	GI:15149310
cIP9	3.3	-0.5	31	0.93	MPSSHLP SFLFPLPSHLLLPSPSLHLLFRKR	Rice	GI:77548735
cIP10	3.0	-0.1	20	0.93	MAKANGSMSSFLMSRKLVAS	Wheat	GI:160701016
cIP11	3.0	-0.4	33	0.94	MSTVT SCTFLSIRPTVSSAGLLSKKPAAFVSL	<i>Arabidopsis</i>	GI:115481218
cIP12	2.0	-0.3	31	0.92	MAFLLPNLSPSFLLPTGKSLKEKPISNQALS	<i>Arabidopsis</i>	GI:21554298
cIP13	2.0	-0.3	24	0.94	MASVSPSPSCSTIPIPLRRTSFAV	<i>Arabidopsis</i>	GI:20197258
cIP14	4.0	-0.2	32	0.97	MSLTIPANLVLNPRSNKSLTQSVPKSSARFVC	<i>Arabidopsis</i>	GI:25083729
cIP15	4.0	-0.2	32	0.93	MAPLPAAAAAPAFLLSPPGKLPGGKPRLKPS	<i>Arabidopsis</i>	GI:125588698
cIP16	6.0	-0.1	33	0.95	MFVLAISSPSTTLIKPLKRNPNRSPVKILCL	<i>Arabidopsis</i>	GI:145361197
cIP17	5.0	0	35	0.99	MAGAAAASSAACSSPAPNPTTRYVRRRVPRPPLV	<i>Arabidopsis</i>	GI:115477777
cIP18	2.0	-0.3	22	0.95	MAPSSFLPANPAGTTARFPVR	Maize	GI:126633352
cIP19	3.0	-0.2	23	0.98	MAVGCNPNVLSPLVPSPGRRVR	Maize	GI:195644780
cIP20	2.0	-0.4	29	0.90	MAGLFASMAVKWAIDKLSLLTPVRQTPV	Rice	GI:77550935
cIP21	2.0	-0.4	19	0.92	MSFPLPSQLSLFLIKPSPR	Rice	GI:22831012
cIP22	3.0	-0.1	28	0.93	MAMPSVGIAALSSTSIATPPRPRRQGPV	Rice	GI:12558527
cIP23	2.1	0	24	0.92	MSSSKQPLLTTVSSTHDGKKMTIL	Wheat	GI:160702683

### **2.2.3 MESOPHYLL PROTOPLAST ISOLATION AND PURIFICATION**

Embryonal halves of triticale (cv. AC Ultima) seeds were surface sterilized with 4% hypochlorite for 1 minute and rinsed three times with sterile water. The embryonal halves were then inoculated on basal Murashige and Skoog (MS) media, pH 5.82 (Murashige and Skoog, 1962). Peeled leaves from six-day-old seedlings were incubated in enzyme solution (2% cellulase and 2% macerozyme, Yakult Honsha Co Ltd, Japan) in cell protoplast washing (CPW) solution, pH 5.6 (Frearson et al., 1973) for 4 hours at 25°C, in the dark.

Protoplasts were isolated by centrifugation at 100 g for 3 minutes, at room temperature (Eppendorf centrifuge 5810R, USA), washed twice with CPW solution and purified by layering on 21% sucrose in CPW solution. A band of protoplasts formed at the interphase and was carefully removed and suspended in CPW solution. After two washings with CPW solution, protoplast density was adjusted to  $2 \times 10^5$  protoplasts/ml.

### **2.2.4 MICROSPORE ISOLATION AND PURIFICATION**

Isolation of triticale (cv. AC Ultima) microspores at the mid-late uninucleate stage were isolated from surface sterilized anthers in NPB-99 media, pH 7.0 (Eudes and Amundsen, 2005).

### **2.2.5 INCUBATION OF ISOLATED TRITICALE PROTOPLASTS OR MICROSPORES WITH FLUORESCENTLY LABELLED PEPTIDES**

Mesophyll protoplasts or microspores (500  $\mu$ l of  $2 \times 10^5$  cells/ml preparation) were incubated with 180  $\mu$ l fluorescently labelled cTPs (100  $\mu$ M) for 1 hour in the dark at

room temperature, followed by washing with CPW solution for protoplasts or NPB-99 media for microspores. The isolated cells were then treated with trypsin-EDTA (0.25%, Sigma-Aldrich) in CPW solution for protoplasts or NPB-99 media for microspores (1:4) for 5 minutes followed by washing with CPW solution for protoplasts or NPB-99 media for microspores and final suspension in CPW solution (500  $\mu$ l) for protoplasts or NPB-99 media (500  $\mu$ l) for microspores.

### **2.2.6 CONFOCAL MICROSCOPY**

Cells were observed using a confocal microscope (Nikon C1, Nikon Canada Inc.) to analyze the subcellular localization of the fluorescently labelled cTPs (excitation wavelength 490 nm / emission wavelength 520 nm). Chloroplasts were identified using chloroplast autofluorescence emission wavelength 633 nm within cells. Fluorescence emissions were collected in z-confocal planes of 10–15 nm and analyzed using EZ-C1 Software Version 3.6. Three-dimensional images were acquired using the following settings on Nikon EZ-C1 software (Gold version 2.3; Image Systems Inc., Columbia, MD): Nikon plan apoc-hromatic 100 X A oil objective (1.49 numerical aperture), 3.6- $\mu$ s scan dwell time, 512 X 512 pixel size resolution, 0.30  $\mu$ m z-step, and a 150- $\mu$ m detector pinhole. The images were analyzed for 3D colocalized fluorescence between the fluorescently labelled peptides and chloroplast autofluorescents.

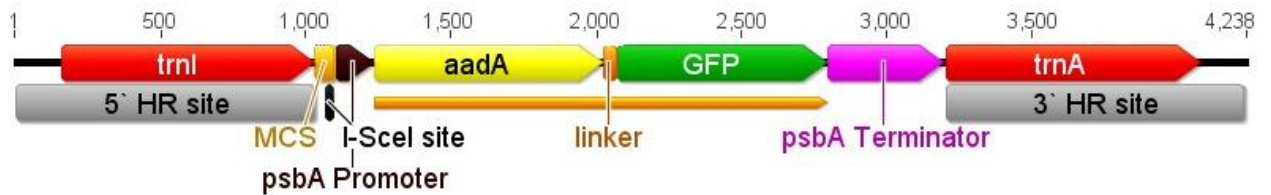
### **2.2.7 REPORTER GENE CONSTRUCT FOR EXPRESSION IN PLASTIDS**

Wheat chloroplast aadA:gfp reporter cassette is a 4238 base pair wheat specific chloroplast transfection vector, designed to target insertions in between the trnI-trnA

inverted repeat region of the *Triticum aestivum* plastid genome (GenBank accession No. AB042240.3), (Fig. 2.1). The vector was designed using Vector NTI® and synthesized by GeneArt®. The nucleotide sequences 92699-93727 and 93792-94822 (GenBank accession No. AB042240.3) were used as the left and right homologous recombination sequences. These sequences share a significant amount of homology with the other trnI-trnA inverted repeat region. A multiple cloning site was introduced following the trnI target insertion sequence. The *aadA:gfp* fusion gene, acted as a dual selection method of spectinomycin / streptomycin resistance through an *aadA* derivative and visual detection by green fluorescent protein (GFP) fluorescence (GenBank accession No. ABX39486) (Khan and Maliga, 1999). The selection marker gene was driven by the *Triticum aestivum* plastid genome promoter (*psbA*) (a core protein of photosystem II) at nucleotides 1282-1153 (GenBank accession No. AB042240.3). The *aad:gfp* fusion gene is terminated with the rice *psbA* terminator sequence derived from chloroplast transformation vector pVSR326 nucleotides 4014-4387 (GenBank accession No. AF527485.1). To test that the construct produced the *aadA:gfp* protein the 6604 base pair wheat chloroplast *aadA:gfp* reporter plasmid was transformed into *E.coli* Rosetta cells according to Novagen® protocol. PCR was used to confirm the plasmid was present within the cells. The cells were also observed using confocal microscopy to confirm GFP expression (Fig. 2.2).

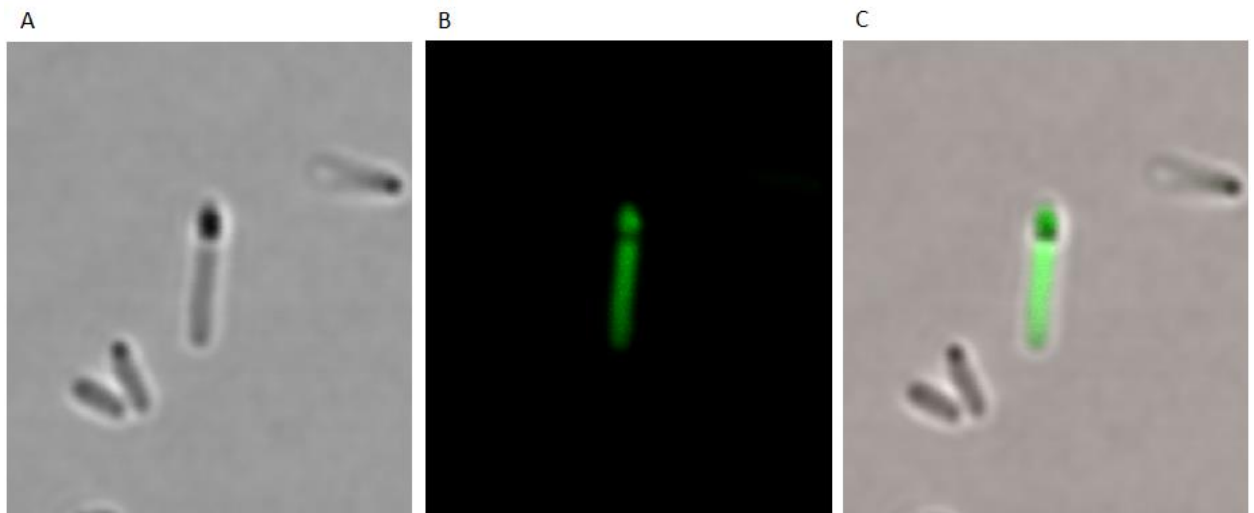
### **2.2.8 GEL MOBILITY SHIFT ASSAY**

The gel mobility shift assay was used to determine the minimum peptide concentration needed to bind to linearized plasmid DNA and cause it to shift during electrophoresis. Purified linearized plasmid DNA (100 ng of linear double stranded DNA,



**Fig. 2.1: A map of the *aadA:gfp* reporter cassette.**

5' HR site = homologous recombination sequence from wheat chloroplast *trnI* gene. MCS = multiple cloning site. psbA promoter = wheat chloroplast psbA promoter. *aadA* = aminoglycoside-3'-adenylyltransferase spectinomycin / streptomycin resistance gene. Linker = 16 amino acid linker between the *aadA* and *gfp* genes. GFP = green fluorescent protein (*gfp*) gene. psbA Terminator = rice psbA terminator. 3' HR site = homologous recombination sequence from wheat chloroplast *trnA* gene.



**Fig. 2.2: Confocal images of *E.coli* Rosetta cells transformed with wheat chloroplast *aadA:gfp* reporter plasmid.**

A) Differential interference contrast (DIC) channel showing transformed *E.coli* Rosetta cells. B) Green channel showing transformed *E.coli* Rosetta cells. C) A composite image of the DIC (*E.coli* Rosetta cells) and green (GFP) channels showing the *aadA:gfp* reporter plasmid is expressing the green GFP protein within the *E.coli* Rosetta cells.

6.8 kb) was mixed with increasing concentrations of each of the cTPs 1-5 listed in (Table 2.1), according to calculated peptide:DNA charge ratios (charge of peptide / charge of DNA 0:1, 1:1, 2:1, 3:1, 4:1, 5:1, to a maximum of 2050:1). To perform the peptide:DNA charge ratio calculation we considered that 1  $\mu$ g of DNA contains 3.03 nM of phosphate and therefore has -3 nM of charge; based on the charge and molecular weight of each peptide it was calculated how much peptide would be required to neutralize the DNAs charge on an integer basis. DNA, 6.8 kb) was mixed with increasing concentrations of each of the cTPs 1-5 listed in (Table 2.1), according to calculated peptide:DNA charge ratios (charge of peptide / charge of DNA 0:1, 1:1, 2:1, 3:1, 4:1, 5:1, to a maximum of 2050:1). To perform the peptide:DNA charge ratio calculation we considered that 1  $\mu$ g of DNA contains 3.03 nM of phosphate and therefore has -3 nM of charge; based on the charge and molecular weight of each peptide it was calculated how much peptide would be required to neutralize the DNAs charge on an integer basis. A reaction containing only plasmid DNA was included as a negative control, which is indicated by the ratio 0:1. The DNA was prepared to a final concentration of a 100 ng/ $\mu$ l in sterile water. Each reaction had a final volume of 25  $\mu$ l and was incubated for 30 minutes, and then subjected to electrophoresis on 1% agarose gel stained with ethidium bromide (Sambrook and Russell, 2006). This procedure was repeated until a complete shift was observed in the plasmid DNA or a maximum peptide:DNA charge ratios of 2050:1 was reached. A maximum peptide:DNA charge ratios of 2050:1 was chosen because higher concentrations of peptide would translate into requiring too much peptide in the transfection reaction and would have made it financially prohibitive.

### **2.2.9 NUCLEASE PROTECTION ASSAY**

The first five cTPs listed in Table 2.1 were separately mixed with linearized plasmid DNA as described for the gel mobility shift assay. For the nuclease protection assay, 5 µl of DNase I (RNase-free DNase set; Qiagen, Valencia, CA, USA) was added to the mixture volume (50 µl). The mixture was incubated at room temperature for 15 minutes and then incubated on ice for 5 minutes. A reaction containing only linearized plasmid DNA was included as a negative control. Plasmid-peptide dissociation and plasmid purification was carried out with a commercially available DNA purification kit (QIAquick™ PCR purification kit; Qiagen). DNA was eluted in sterile water. An aliquot of 6 µl was subjected to 1% agarose gel electrophoresis.

### **2.2.10 PROTOPLAST AND MICROSPORE TRANSFECTION WITH *aadA:gfp* REPORTER**

The linearized dsDNA cassette (*aadA:gfp* for chloroplast expression, 1.5 µg) was combined with 1 of each of the 5 cTPs (scaled up from 100 ng, at 4 times the concentration needed to cause a DNA shift in the gel mobility shift assay) in a final volume of 100 µl in CPW solution for protoplasts or NPB-99 media for microspores. The mixture is incubated for 10 minutes at room temperature, and then incubated with isolated triticale mesophyll protoplasts (500 µl of  $2 \times 10^5$  protoplasts/ml) for 1 hour in the dark at room temperature. CPW solution for protoplasts or NPB-99 media for microspores (500 µl) was added and the mixture was incubated in the dark for 24 hours. The cells were imaged using confocal microscopy, focusing on chloroplast autofluorescence to identify plastids.

### **2.2.11 DNA AND RNA EXTRACTION OF TRANSFECTED PROTOPLASTS, MICROSPORES AND PLANT TISSUE**

Following a 48 hours, 21°C incubation, transfected protoplasts, microspores or regenerated plant tissue were frozen at -80°C. These frozen samples were disrupted using ceramic beads and aggressive shaking for 1 minute in a 2.0 ml microcentrifuge tube. An aliquot of 450 µl of RLT buffer/B-Me from Qiagen's All Prep DNA/RNA mini kit was added to the sample and then it was vortexed for 10 seconds. The samples were heated at 55°C for 1 minute and vortexed for 10 seconds again. Extraction of the DNA and RNA was performed using Qiagen's All Prep DNA/RNA mini kit using the on column DNase I digestion according to the manufacturer's protocol. The final elution volume was 40 µl. DNA and RNA quantity and quality was assessed using an agarose gel (to ensure DNA and RNA integrity) and quantified spectrophotometrically.

### **2.2.12 REVERSE TRANSCRIPTION PCR (RT-PCR)**

cDNA was generated using a SuperScript<sup>®</sup> III First-Strand Synthesis Kit (Life Technologies). One µg of RNA was added to each reaction and the manufacturer's protocol for random priming was followed, with the exception that the volume of the reaction mixture was increased from 20 µl to 30 µl. A negative reverse transcription (RT) sample containing all reagents but no RNA was included in the cDNA synthesis as a control.

All real time PCR reactions were performed using QuantiTect<sup>™</sup> SYBR<sup>™</sup> Green PCR Master Mix (Qiagen) in a 20 µl reaction volume. Each reaction contained 1 µM of each primer GFP4L 5'-acatcacggcagacaaaca-3' and GFP4R 5'-aaagggcagattgtgtggac-3' and was tested in triplicate using 3 µl of template in each reaction. Cycling was



performed as follows: 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds. Dissociation curves were run after the PCR reactions were completed. The 3 CT values were averaged for each sample and a standard deviation was calculated. Standard curves were generated by plotting average CT value against the log of the amount of DNA in each sample so that the PCR efficiency of each primer set could be assessed. The primer sets were amplified with comparable efficiency so CT values could be directly compared.

Standard curves were generated for both elongation factor 1 alpha (*EF1 $\alpha$* ) (housekeeping control) and *aadA:gfp* genes. The *EF1 $\alpha$*  curve was generated by pooling 6  $\mu$ l of each cDNA sample (excluding the negative reverse transcription control), then carrying out a two fold serial dilution five times. The GFP standard curve was generated using the plasmid DNA used for transfection (13 ng/ $\mu$ l plasmid, of which 3  $\mu$ l (40 ng) were used for the PCR reaction, followed by a two fold serial dilution five times).

Relative expression levels of *aadAGFP* /*EF1 $\alpha$*  were measured using the outlined RT-PCR method. The mean relative expression levels of three protoplast transfections performed using each of the five cTP peptides delivering the *aadAGFP* reporter cassette were analyzed by one-way ANOVA using SPSS 15.0 software. P values ( $P \leq 0.05$ ) were utilized to determine if the measured changes in transcript levels were significant.

### **2.2.13 MICROSPORE TRANSFECTION WITH REPORTER CASSETTE AND PLANT PROPAGATION**

To test the application of plastid transfection in the microspore tissue culture system using cTP1 we applied the previously described microspore transfection protocol to genetically engineer the plastid genome of triticale microspores. Following the 1 hour

incubation after the microspore transfection, 100 mg/L of streptomycin was added to the induction media, and cultured in the dark at 27°C. At 4 weeks developing embryos were transferred to a Rita Box semi-immersion culture system using 200 ml of liquid GEM supplemented with 200 µl PPM™, beneath Sylvania GRO-LUX® wide spectrum bulbs (40 watts) delivering 80 µM m<sup>-2</sup> s<sup>-1</sup> (16 hour light period) with a room temperature at 16°C. The following streptomycin selection regime was applied to the developing embryos (week 1 - 4, 100 mg/L; week 5, 200 mg/L and week 7, 400 mg/L streptomycin selection). At 9 weeks, germinated (green and variegated) plantlets were transferred to soil, in root trainers. The streptomycin selective pressure was not maintained in soil. The microspore control cultures were carried out in absence of transfecting peptide or DNA. Two sets of microspore control cultures were prepared, 1 set containing streptomycin and the other without to determine the effect antibiotics would have on green plant regeneration.

#### **2.2.14 MOLECULAR EVALUATION OF T<sub>0</sub> PLANTLETS**

Genomic DNA was isolated from 5 cm young leaves of the primary transformants (T<sub>0</sub>) at the end of the *in vitro* culture steps, using DNeasy Plant Mini Kit (Qiagen). To determine which plants were positive for the reporter, PCR was performed with 2 µl of template in a final volume of 20 µl. Each reaction contained 1 µM of each primer GFP1L 5'-cacggcagacaaacaaaaga-3' and GFP1R 5'-aaagggcagattgtgtggac-3'. Cycling was performed as follows: 94°C for 3 minutes, 30 repeats of 94°C for 1 minute, 60°C for 30 seconds, 72°C for 30 seconds, final extension of 72°C for 10 minutes. Samples were separated by electrophoresis on a 1.0% agarose gel in 1× TAE buffer for 1 hour.

## 2.3 RESULTS

### 2.3.1 cTP PEPTIDE SELECTION AND EVALUATION

The total number of Genbank plant protein sequences downloaded 785 615 was reduced to 349 when TargetP predicted cTP sequences were parsed sequentially and cumulatively using the selection criteria of TargetP relative confidence value of  $\geq 0.9$ , a sequence length of  $\leq 35$  aa and an average hydrophilicity  $\leq 0$  (Table 2.2). Twenty three candidate of the 349 TargetP predicted cTP sequences, defined as having a charge ratio of above 2 and a hydrophilicity of  $\leq 0$  were selected and synthesized (Table 2.1). Each peptide was labelled with FITC at the N-terminus, to facilitate visual detection by fluorescence and then tested for cell penetrating and plastid targeting properties using confocal microscopy to determine what organelle the peptide co-localized to in protoplasts.

There was a large range of ability to transduce across the outer plasma membrane and specifically targeting the chloroplast among the tested peptides. Two peptides exhibited weak diffuse fluorescents within the cytosol, three peptides were observed to be contained within endosomes, eight peptides exhibited nonspecific localization around the surface of the chloroplasts but not enter into the chloroplasts and ten were observed to be contained within endosomes as well as aggregate at the chloroplast surface but again not enter into the chloroplasts (data not shown). Out of the twenty three peptides tested, five cTPs were found to both penetrate the outer plasma membrane and specifically localize to the chloroplast (Fig. 2.3, 2.4 and Table 2.1). Figure 2.3 is a single slice cross section of a protoplast transfected with fluorescently labelled cTP1. The normalization of the image from the background subtracted green and red channel negative control protoplast sample ensured that any fluorescents observed in the image was that of the fluorescently labelled

peptide and not chloroplast autofluorescents. Compiling multiple single slice cross sectional images of protoplast samples transfected with one of the five cTPs to generate 3D images using the confocal microscopes software I was able to manually manipulate the 3D and cross sectional images to confirm that the observed fluorescent was localized within the chloroplast as opposed to just around the chloroplasts outer surface. This demonstrates that cTP1 crossed the outer plasma membrane and the chloroplast double membrane, specifically localizing within the stroma of the chloroplasts. The same method and similar observations were made for cTPs 2-5 with the peptides having crossed the outer plasma membrane with the majority of the peptides localizing at the chloroplasts outer membrane and a small concentration of the peptides being observed to cross the chloroplasts inner membrane localizing in the stroma like cTP1 when compared to the negative control (Fig 2.3 and 2.4). The five cTP peptides having both cell penetrating and chloroplast targeting properties in protoplasts (cTP1-cTP5, Table 2.1) were also tested in isolated triticale microspores. It was determined that cTPs accumulate within microspores due to the large fluorescent signals observed over the corrected background fluorescent levels. This demonstrates that the cTPs have the ability to translocate across the isolated microspore plasma membrane which is surrounded by layers of exine and intine. We could not conclude exactly where the cTPs were specifically localizing in the transfected microspores because there was no autofluorescence emitted by the proplastids within the microspores.

### **2.3.2 cTP PEPTIDE DNA BINDING AND PROTECTION ANALYSIS**

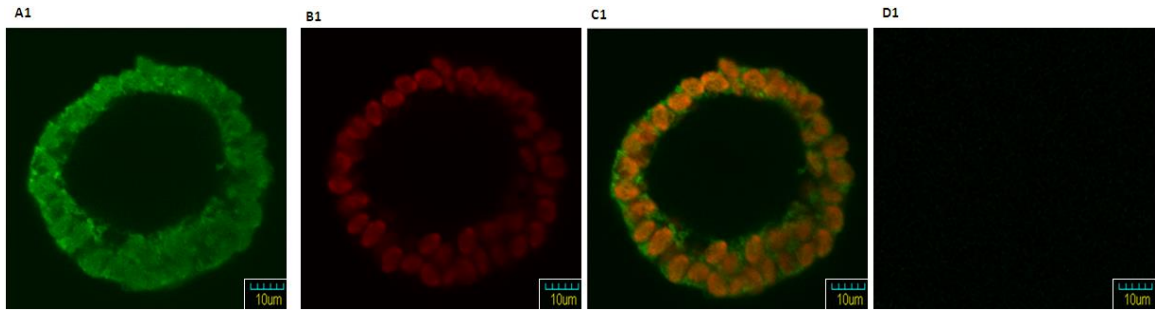
To determine if cTPs 1-5 could be utilized to deliver cargo into cells, the peptides were tested for their ability to non-covalently bind to nucleic acids. A gel mobility shift

assay and a nuclease protection assay were separately performed with each peptide to determine the minimum peptide concentration needed to bind to and completely saturate the linearized dsDNA in preparation for the protoplast and microspore transfections. Three of the peptides (cTP1, cTP2 and cTP5) caused a mobility shift in the DNA during electrophoresis (Fig. 2.5 – 2.7). The peptide:DNA molar binding ratios varied from 16.3 M for cTP1, 15.0 M for cTP2 and 6340.0 M for cTP5 (Table 2.3). The variation in the amount of peptide needed to cause a shift in the DNA during electrophoresis could relate to the peptides overall charge. Peptide cTP2 having the highest charge of 4.2, required the least amount of peptide needed to cause a shift and cTP5 having a charge of 2 required the largest amount of peptide needed to cause a shift. This indicates that peptides with higher amounts of positive charge require a lower concentration of peptide needed to neutralize the negative charge of the DNA and cause a shift in the DNA during electrophoresis. Another contributing factor to the variation in the amount of peptide needed to cause a shift in the DNA during electrophoresis could relate to variation in amino acid sequence as well. The observed band shift could also be an artifact of the peptides interfering with the migration of the DNA through the agarose gel and not the peptide binding to the nucleic acids. The inability of cTP3 and cTP4 to cause a mobility shift in the DNA during electrophoresis maybe due to variation in amino acid sequence and a lack of sufficient positive charge within the peptides to form a stable non-covalently bond between the negatively charged DNA and the positively charged peptides, in the presence of an electric field (Table 2.3). A factor to consider when introducing nucleic acids into the cytosol of a cell is nuclease degradation of the nucleic acids. Therefore to determine if the cTP peptides are capable of protecting DNA from nuclease degradation a nuclease protection assay was performed. The nuclease protection assay determined that

**Table 2.2. Chloroplast transit peptide sequence selection.**

The first 3 columns of numerical data in Table 2.2, represent the total number of starting protein sequences from NCBI GenBank from each organism, the total number of cTP sequences predicted by TargetP and the predicted number of cTP sequences with a TargetP relative confidence level of  $\geq 90\%$ , respectively. The remaining columns of numerical data represent the numbers of sequences predicted when the following selection criteria were applied sequentially and cumulatively: a sequence length of 35 aa or less, a net positive charge of  $\geq 3.5$  and an average hydrophilicity of  $\leq 0$ .

Species	Total starting protein sequences	Total cTP Sequences	Relative Confidence Level $\geq 0.9$	Sequence Length $\leq 35$	Net Positive Charge $\geq 2.0$	Average Hydrophilicity $\leq 0$
<i>Arabidopsis</i>	387 781	41 132	5 016	503	299	193
Rice	302 221	40 830	6 571	476	258	115
Maize	60 709	9 898	904	78	52	33
Wheat	34 904	2 931	373	86	31	8
Totals	785 615	94 791	12 864	1 143	640	349

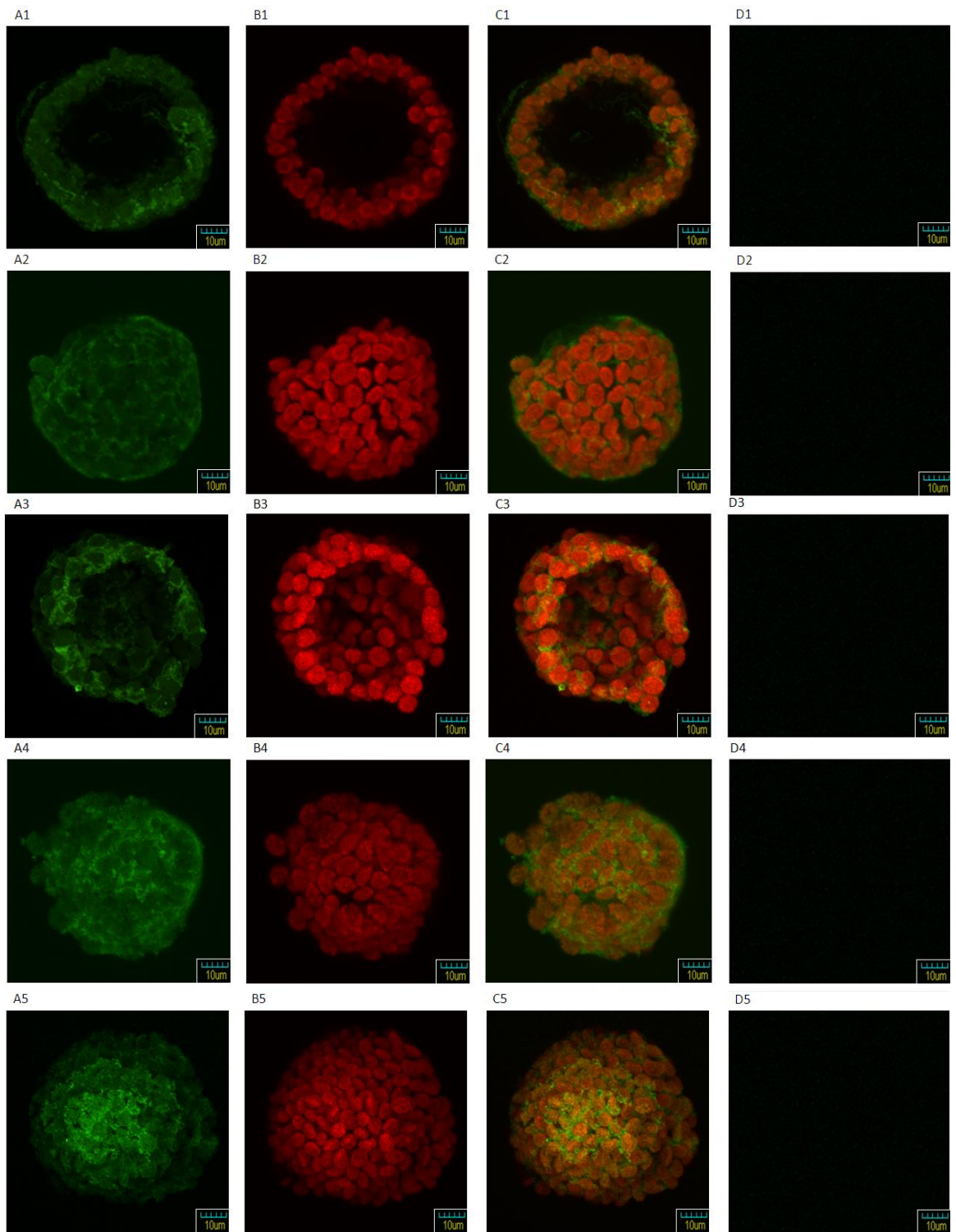


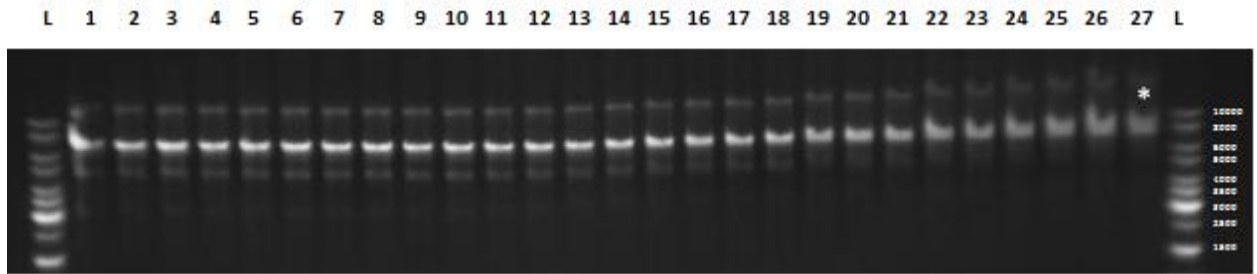
**Fig. 2.3: Single slice cross section confocal images of a FITC labelled cTP1 transfected protoplast.**

A1) Green channel showing the FITC labelled cTP1 peptide has transduced across the outer plasma membrane of the transfected protoplast. B1) The red channel showing chloroplast autofluorescence. C1) A composite image of the red (chloroplasts) and green (peptide) channels. These images show the co-localization of the green fluorescently labelled peptide with the red chloroplast autofluorescence. D1) Negative control image of protoplasts with the background subtracted green and red channels.

**Fig. 2.4: Confocal images of a FITC labelled cTPs 1-5 transfected protoplasts.**  
A1-5) Green channel showing the FITC labelled cTP1-5 peptide has transduced across the outer plasma membrane of the transfected protoplast. B1-5) The red channel showing chloroplast autofluorescence. C1-5) A composite image of the red (chloroplasts) and green (peptide) channels. These images show the co-localization of the green fluorescently labelled peptide with the red chloroplast autofluorescence. D1-5) Negative control image of protoplasts with the background subtracted green and red channels.



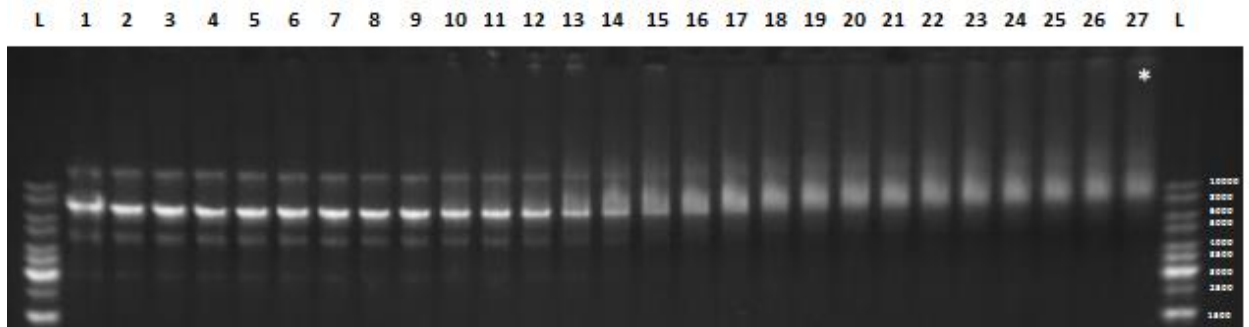




**Fig. 2.5: Gel mobility shift assay of linearized plasmid DNA incubated with cTP1.**

The linearized plasmid DNA was packaged with various molar concentrations of peptide that correspond to calculated peptide:DNA charge ratios. L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) 1:1, 2) 10:1, 3) 15:1, 4) 20:1, 5) 25:1, 6) 30:1, 7) 35:1, 8) 40:1, 9) 45:1, 10) 50:1, 11) 55:1, 12) 60:1, 13) 70:1, 14) 80:1, 15) 90:1, 16) 100:1, 17) 125:1, 18) 150:1, 19) 175:1, 20) 200:1, 21) 225:1, 22) 250:1, 23) 275:1, 24) 300:1, 25) 325:1, 26) 350:1, 27) 375:1.

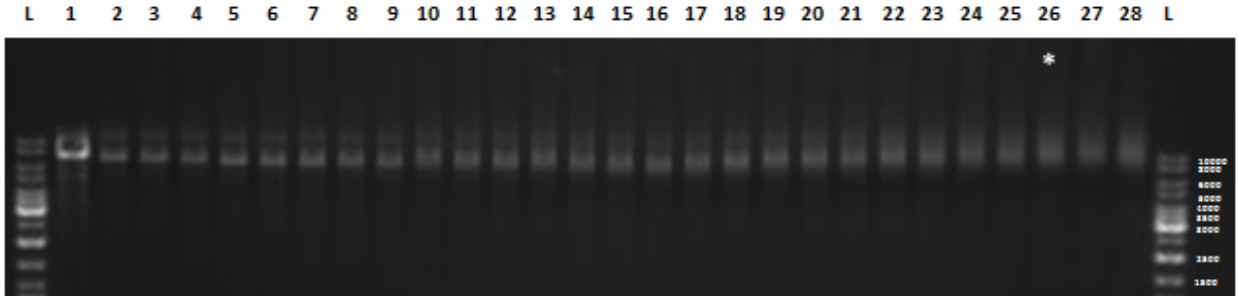
\* Indicates the point where a complete shift was observed in the plasmid DNA. The molar ratio of peptide:DNA was 16.3:1.



**Fig. 2.6: Gel mobility shift assay of linearized plasmid DNA incubated with cTP2.**

The linearized plasmid DNA was packaged with various molar concentrations of peptide that correspond to calculated peptide:DNA charge ratios. L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) 1:1, 2) 10:1, 3) 20:1, 4) 30:1, 5) 40:1, 6) 50:1, 7) 60:1, 8) 70:1, 9) 80:1, 10) 90:1, 11) 100:1, 12) 125:1, 13) 150:1, 14) 175:1, 15) 200:1, 16) 225:1, 17) 250:1, 18) 275:1, 19) 300:1, 20) 325:1, 21) 350:1, 22) 375:1, 23) 400:1, 24) 425:1, 25) 450:1, 26) 475:1, 27) 500:1.

\* Indicates the point where a complete shift was observed in the plasmid DNA. The molar ratio of peptide:DNA was 15.0:1.

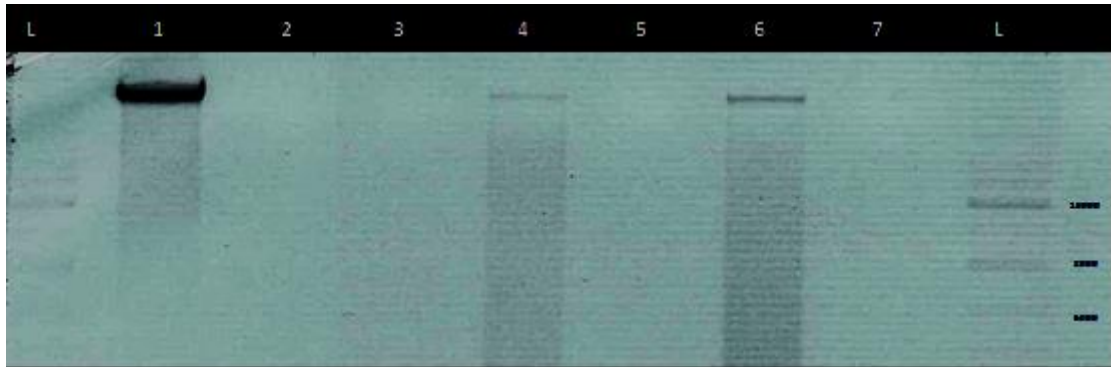


**Fig. 2.7: Gel mobility shift assay of linearized plasmid DNA incubated with cTP5.** The linearized plasmid DNA was packaged with various molar concentrations of peptide that correspond to calculated peptide:DNA charge ratios. L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) 1:1, 2) 750:1, 3) 775:1, 4) 800:1, 5) 825:1, 6) 850:1, 7) 875:1, 8) 900:1, 9) 925:1, 10) 950:1, 11) 975:1, 12) 1000:1, 13) 1025:1, 14) 1050:1, 15) 1075:1, 16) 1100:1, 17) 1125:1, 18) 1150:1, 19) 1175:1, 20) 1200:1, 21) 1225:1, 22) 1250:1, 23) 1275:1, 24) 1300:1, 25) 1325:1, 26) 1350:1, 27) 1375:1.  
\* Indicates the point where a complete shift was observed in the plasmid DNA. The molar ratio of peptide:DNA was 6340:1.

**Table 2.3. Gel shift and nuclease protection assay experimental results.**

The table summarizes the cTP gel shift and nuclease protection assay experimental results.

<b>Peptide</b>	<b>Peptide:DNA Molar Binding Ratio</b>	<b>Nuclease Protection</b>
<b>cTP1</b>	<b>16.3:1</b>	<b>+</b>
<b>cTP2</b>	<b>15.0:1</b>	<b>+</b>
<b>cTP3</b>	<b>-</b>	<b>-</b>
<b>cTP4</b>	<b>-</b>	<b>-</b>
<b>cTP5</b>	<b>6340:1</b>	<b>-</b>



**Fig. 2.8: Nuclease protection of 100 ng of linearized plasmid DNA by cTPs 1-5.**  
L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) Linearized plasmid DNA. 2) Linearized plasmid DNA plus DNase I. 3) Linearized plasmid DNA plus cTP3 and DNase I. 4) Linearized plasmid DNA plus cTP1 and DNase I. 5) Linearized plasmid DNA plus cTP5 and DNase I. 6) Linearized plasmid DNA plus cTP2 and DNase I. 7) Linearized plasmid DNA plus cTP4 and DNase I.

cTP1 and cTP2 were able to protect the DNA from nuclease degradation at or above the peptide:DNA molar ratios required to cause a mobility shift in the DNA using the gel mobility shift assay (Fig. 2.8 and Table 2.3).

### **2.3.3 cTPs CAN BE USED TO DELIVER A DNA REPORTER CASSETTE TO PLASTIDS IN TISSUE CULTURED PLANT CELLS**

The peptide reporter cassette transfection assay was used to determine if the five candidate peptides could be used to transfect the *aadA:gfp* reporter cassette into triticale mesophyll protoplast or microspore plastids. Following transfection, the triticale mesophyll protoplast chloroplasts were observed to fluoresce in both the green and red background subtracted channels. The co-localization of the two fluorescent signals indicates that the cTP1 *aadA:gfp* reporter cassette transfected protoplasts were expressing

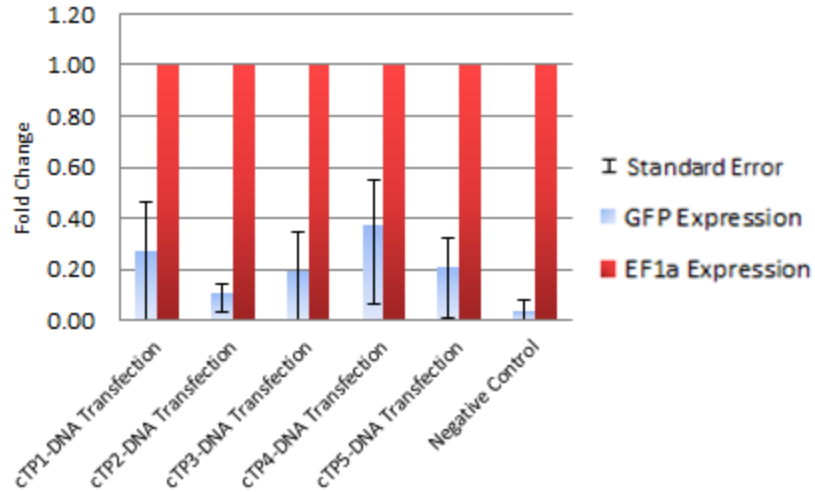
### **2.3.4 EXPRESSION ANALYSIS OF cTP *aadA:gfp* CASSETTE TRANSFECTED PLANT CELLS**

Quantitative real-time PCR (qRT-PCR) analysis being more sensitive than confocal imaging was performed to detect and quantify transcription of the *aadA:gfp* reporter cassette used in the transfection of the microspores and protoplasts plastids. The fold difference of normalized *aadA:gfp* mRNA expression, over the housekeeping control elongation factor 1 alpha mRNA expression level, ranged from 0.08 to 0.31 fold in microspores to 23.86 to 121.95 fold in protoplasts (Fig. 2.9 and 2.10). The high variation in CT values in the RT-PCR used to analyze each of the three microspore and protoplast transfections performed for each of the five cTPs resulted in high p-values > 0.05 when analysed using the One-way ANOVA method. This means that the transfected

microspores and protoplasts were expressing the *aadA:gfp* gene but the mean of the three cTP transfected microspore and protoplast samples CT values measured using RT-PCR weren't significantly different from the negative control.

### **2.3.5 GENERATION OF TRITICALE PLANTS CONTAINING GENETICALLY ENGINEERED PLASTIDS**

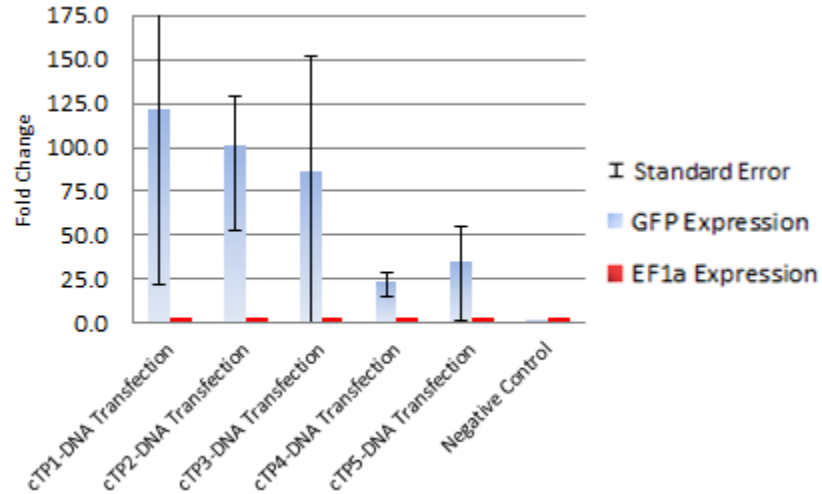
Transgenic plants were generated to demonstrate that the peptide transfection method could be utilized to stably integrate a transgene into the plastid genome and produce androgenic haploid or double haploid plants with a homoplasmic population of transgenic plastids. Isolated microspore culture (IMC) was used for this purpose because this method is compatible with the *in vivo* application of our peptide transfection method and selection protocol and it circumvented the recalcitrance of fertile green plant production from protoplasts. Isolated microspore culture is primarily used to increase the homozygosity of crop plants such as triticale in breeding programs but also increased the probability of producing a fertile homoplasmic plant due to the reduced number of proplastids contained within microspores when compared to somatic cells (José and Fernando, 2008; Matsushima et al., 2008). To test if the plastids of green plants could be genetically engineered, cTP1 was used to transfect isolated triticale microspores with the *aadA:gfp* reporter cassette. Neither peptides nor DNA were added to the microspore culture control reactions but to determine the effects of streptomycin selection on green plant regeneration in microspore culture, we included a microspore culture control with streptomycin selection and one without. Two green plants with streptomycin selection were regenerated from seven microspore culture control reactions. Control plants without streptomycin selection were regenerated but most of them were albino. The green plants that were produced without streptomycin selection died when transferred to soil.



**Fig. 2.9: Quantitative real-time PCR (qRT-PCR) analysis of cTP-DNA transfected microspores.**

Quantitative real-time PCR (qRT-PCR) analysis was used to confirm that the plastids of cTP-DNA transfected microspores were expressing GFP. The blue bars represent *GFP* mRNA expression levels. The red bars represent *EF1 $\alpha$*  mRNA expression levels. Black standard error bars illustrate the variation of the mean between the three transfected samples. The fold difference of normalized *GFP* mRNA expression compared to the internal control *EF1 $\alpha$*  mRNA expression levels.





**Figure 2.10: Quantitative real-time PCR (qRT-PCR) analysis of cTP-DNA transfected protoplasts.**

Quantitative real-time PCR (qRT-PCR) analysis was used to confirm that the plastids of cTP-DNA transfected protoplasts were expressing GFP. The blue bars represent *GFP* mRNA expression levels. The red bars represent *EF1 $\alpha$*  mRNA expression levels. Black standard error bars illustrate the variation of the mean between the three transfected samples. The fold difference of normalized *GFP* mRNA expression compared to the internal control *EF1 $\alpha$*  mRNA expression levels.

Therefore only the two control plants produced with streptomycin selection were available for analysis.

### **2.3.6 MOLECULAR ANALYSIS OF TRITICALE PLANTS CONTAINING GENETICALLY ENGINEERED CHLOROPLASTS**

Genomic DNA was isolated from the leaves of the twelve primary transformants (T<sub>0</sub>) produced from microspores transfected with cTP1 complexed with the *aadA:gfp* reporter cassette and two microspore derived control plants produced with streptomycin selection. This DNA was screened using GFP1L and GFP1R PCR primers to evaluate which of the regenerated plants contained the *aadA:gfp* gene. Zero out of the twelve plants was characterized as being PCR positive due to the absence of an amplicon of the correct size (data not shown). Plants regenerated from control transfection did not show an amplicon.

## **2.4 DISCUSSION**

To select cTPs with cell penetrating and organelle targeting characteristics we had to identify physical and chemical properties required to cross three cellular membranes; first the outer plasma membrane and then the double membrane of the plastid/chloroplast. Cell penetrating peptides such as Tat have the ability to cross cellular plasma membranes and localize in the nucleus of cells demonstrates that other protein sorting signals such as cTPs could possess similar cell penetrating properties in conjunction to their organelle targeting properties. (Vives et al., 1997; Astriab-Fisher et al., 2002).

Software such as TargetP, ChloroP, Predotar and iPSORT were developed to predict chloroplast subcellular localization signals (Bannai et al., 2002; Small et al., 2004; Emanuelsson et al., 2007). TargetP had the highest positive predictive values when

compared to the previously stated bioinformatics programs at the time of analysis therefore to maximize our chances of predicting true cTP sequences we used this program to analyse all of the downloaded plant protein sequences (Bannai et al., 2002).

The basis for our CPP selective criteria was determined by the physicochemical properties necessary for CPPs to cross membranes. It is a combination of hydrophobicity, high net positive charge and the aa R-groups of arginine and lysine that confer membrane transduction properties to CPPs (Herce and Garcia, 2008). Some of these properties such as hydrophobicity, positive charge and the propensity to form  $\alpha$ -helical structures are shared by both cTPs and CPP (Scheller et al., 1999; Bruce, 2001). The defined physicochemical properties that constitute a CPP or a cTP length, charge, and hydrophobicity were used as the selection criteria and was found to be sufficient in identifying cTPs with CPP properties. Further refinement and the inclusion of other peptide properties such as aa composition and secondary structure should be considered in future functional peptide prediction experiments.

To visually screen the twenty three fluorescently labelled cTPs for protein transduction and organelle targeting properties confocal microscopy was used. Through the combination of background subtracted of the green and red channel from negative control protoplast samples to ensure that any fluorescents observed in the fluorescently labelled cTP transfected sample images were that of the fluorescently labelled peptides and not chloroplast autofluorescents and the analysis of both cross sectional and 3D images I was able to confirm that the observed fluorescents was localized within the chloroplast as opposed to just around the chloroplasts outer surface. This demonstrates that cTP1 crossed the outer plasma membrane and the chloroplast double membrane, specifically localizing within the stroma of the chloroplasts. The same method and similar

observations were made for cTPs 2-5 with the peptides having crossed the outer plasma membrane with the majority of the peptides localizing at the chloroplasts outer membrane and a small concentration of the peptides being observed to cross the chloroplasts inner membrane localizing in the stroma like cTP1 when compared to the negative control (Fig 2.3 and 2.4). Higher throughput methods such as flow cytometry may want to be considered in future experimentation of this type.

In determining if the cTPs had the capacity to be used as a vehicle to deliver DNA to plastids in isolated plant cells. Both the gel shift and nuclease protection assays facilitated the determination of the minimum peptide concentration needed to bind to and completely saturate DNA in preparation for the transfection experiments. It was previously determined that the CPP Tat<sub>2</sub> had the ability to form stable complexes between the peptide and linearized plasmid DNA using a gel mobility shift assay, nuclease protection assay as well as SEM microscopy to visualize the nanoparticles formed between the peptides and DNA (Chugh et al., 2009). Our gel shift and nuclease protection assay data confirmed Chugh et al., (2009) findings with regard to peptide DNA complex formation. Two out of five cTPs (cTP1 and cTP2) have the ability to bind DNA non-covalently forming a nanocomplex that protects DNA from nuclease degradation (Fig. 2.5 – 2.7 and 2.8 and Table 2.3). The observed band shift could also be an artifact of the peptides interfering with the migration of the DNA through the agarose gel and not the peptide binding to the nucleic acids. The fact that not all of the peptides were able to bind to and protect DNA in these assays may indicate that variation in amino acid sequence and cationic charge affects stable non-covalent bond formation between the peptides and DNA. The two cTPs that bound DNA and protected it from nuclease degradation both

had a cationic charge greater than 2.9; this maybe the charge threshold at which the cTP peptides can form a stable complex with DNA under our electrophoresis conditions.

Electrophoretic mobility of protein-nucleic acid complexes can be affected by the conditions (temperature, pressure, pH, salt and ion concentrations) of the assay (Hellman and Fried, 2007). It was demonstrated that the binding of protein to DNA during electrophoresis is pH dependent. The *E. coli* tryptophan repressor protein failed to cause a shift when complexed with DNA containing the tryptophan operator at pH 8.3 but did protect it from nuclease degradation during a nuclease footprinting assay. Changing the pH of the electrophoresis buffers to a pH of 6.0 utilized in the initial experiment resulted in a mobility shift of the protein-DNA complex (Carey, 1988). To further refine the concentration of peptides needed for the transfection reaction or to characterize the peptide DNA complex future work may involve changing the pH and ion concentrations of transfection and electrophoretic buffers.

The vector used in the transfection reactions was the *aadA:gfp* reporter cassette. Cereal crops such as rice and wheat possess an inherent spectinomycin resistance, due to the occurrence of point mutations in their 16S rRNA sequences (Fromm et al., 1987). However, it was demonstrated that the spectinomycin GFP fusion protein expressed well in rice therefore it would facilitate the visual detection of plastid gene expression in triticale also a monocot species (Khan and Maliga, 1999). The *aadA:gfp* fusion gene also presents the opportunity to apply antibiotic selection to the cell culture media for the selection of cells containing transgenic plastids. The *aadA* gene inactivates both spectinomycin and streptomycin. Therefore streptomycin selection was applied as the selective agent to identify cells containing transgenic plastids. An antibiotic resistance gene that has recently been applied to monocot plastid transformation is neomycin

phosphotransferase II (*nptII*); although these results need to be experimentally confirmed by the authors (Cui et al., 2011). Other antibiotic resistance genes that have been used in dicot plastid transformation but have yet to be applied to monocot plastid transformation are chloramphenicol acetyltransferase (*cat*), and aminoglycoside phosphotransferase (*aphA-6*) (Huang et al., 2002; Li et al., 2011). An alternative to antibiotic resistance is the herbicide selectable marker acetolactate synthase gene that has been effectively used in monocot nuclear transformation and dicot plastid transformation experiments could be considered for future monocot plastid transformation experiments (Ogawa et al., 2008; Shimizu et al., 2008).

The *aadA:gfp* reporter cassette was designed with 500 bp of flanking homologous sequence on either side of the reporter cassette to target integration of the transgene into the wheat plastome inverted repeat region (IR) between the *trnI-trnA* genes. The wheat plastome is 134 545 bp in size with two identical copies of a 20 703 bp inverted repeat (IRa and IRb) region (Ogihara et al., 2002). Chloroplasts support homologous recombination and transgenes that recombine into the IR region are often duplicated in a process called copy correction into the opposing IR region (Cerutti et al., 1992; Kavanagh et al., 1999). Increasing the size of the flanking homologous regions to 1-2 kb may increase the chances of homologous recombination at this site if this vector is used in future cereal plastid engineering applications. The inclusion of the *oriA* in the left *trnI* flanking region has been shown to enhance transgene integration, expression and the achievement of homoplasmy (Daniell et al., 1990; Guda et al., 2000). The chloroplast origin of replication *oriA* has been mapped to the *trnI* gene in tobacco and shows a high degree of sequence conservation within monocot species (Kunnimalaiyaan and Nielsen, 1997; McNutt et al., 2007). All of these factors contribute to its popularity and the reason

the *trnI-trnA* site was chosen as our target site. Alternatively, investigation of alternative homologous recombination regions may support higher levels of gene integration.

The five non labelled cTPs 1-5 listed in Table 2.1 were tested for their ability to package and deliver the linear double stranded DNA *aadA:gfp* reporter cassette into the plastids of isolated triticale protoplasts and microspores. By co-localizing the GFP fluorescents with chloroplast autofluorescence signals using confocal microscopy I qualitatively determined that the *aadA:gfp* reporter cassette was being expressed in the transfected protoplasts. Green fluorescent protein expression was insufficient or microspore autofluorescence was too high to directly observe GFP expression within transfected microspores. This low level of expression within microspores was individually measured using RT-PCR but when comparing the mean CT values of the three transfected samples to the negative control they weren't statistically significantly different from the negative control (Fig 2.9). The low levels of expression can be explained by the fact that microspores contain proplastid; a non-photosynthetic organelles that differentiates into chloroplasts and other plastid types organelles during cellular maturation. Proplastids have been characterized as having low levels of DNA copy number, transcription rates and RNA (Mullet, 1993). As proplastids progress through the developmental process plastid transcription and translation is elevated to meet the high protein requirement of mature chloroplasts involved in photosynthesis (Demarsy et al., 2006). This explains the high expression levels measured using RT-PCR in the transfected protoplasts and the difference in expression observed between the two different cell types (Fig 2.9 and 2.10).

Protoplasts are recalcitrant to whole plant regeneration therefore only serve as a model system for the testing and characterisation of the cTPs. Alternatively microspore

culture is amenable to fertile green plant production and presents an embryogenic cell culture system that can be used for monocot plastid genetic engineering. Our lab currently uses wheat and triticale isolated microspore culture in conjunction to a nuclear CPP microspore transfection protocol to create transgenic plants (Chugh et al., 2009). To test the application of cTPs in plastid transfection of microspores we attempted to genetically engineer the plastid genome of triticale microspores. Following transfection of isolated microspores they were induced to become embryogenic and gave rise to multicellular embryos that were regenerated under streptomycin selection into mature plants (José and Fernando, 2008). None of the mature plants tested positive for the transgene when screened using PCR. This may indicate our selective pressure wasn't high enough or a different selectable marker should be used in the future. In future monocot plastid genetic engineering experiments one might consider the use of herbicide selectable markers such as acetolactate synthase gene that have been effectively used in monocot organelle transformation experiments (Ogawa et al., 2008).

In the pursuit of achieving transgenic plastid homoplasmy in monocot species it will require identifying an extremely efficient selection protocol that eliminates all wild type (wt) plastid DNA in the first round of plant regeneration or the development of plant tissue culture methods that allows for multiple rounds of selective tissue culture to dilute and eliminate wt plastid DNA during plant regeneration. These methods have proved to be successful in achieving transplastomic homoplasmy in dicot crop species but have only seen limited success in monocots (Daniell et al., 2005). Despite the current technical difficulties in monocot plastid transformation our peptide based plastid transfection method could be an alternative strategy for future monocot transplastomic investigations and technology development if the technology is optimized.



### **3.0 PLANT MITOCHONDRIAL-TARGETING CELL PENETRATING PEPTIDES.**

#### **3.1 INTRODUCTION**

Having the ability to genetically engineer plant cell mitochondria has many important applications in cell biology and agricultural biotechnology. Mitochondria are present in essentially all eukaryotic organisms including plants, animals, protists, fungi and protozoa. Mitochondria play a central role in animal and plant cellular metabolism. They regulate the production of ATP through oxidative phosphorylation, carbon and nitrogen metabolism in the tricarboxylic acid cycle and stress responses through the alternative oxidase pathway (Millar et al., 2011). Mitochondria have also been linked to agronomically important plant traits such as cytoplasmic male sterility which has been applied to crop breeding and hybrid maintenance (Frei et al., 2010). It is critical to gain a greater understanding of the mitochondria's roll in all of these essential metabolic pathways and how this knowledge can be applied to altering photosynthetic rates, biomass production, secondary metabolite accumulation, disease resistance, nitrogen assimilation and plant breeding.

Currently mitochondrial research in both plant and animal cells is limited due to the inability to manipulate the mitochondria genome in whole cells. *Chlamydomonas reinhardtii* and two yeast species, *Saccharomyces cerevisiae* and *Candida glabrata* mitochondria have been transformed using the biolistic method (Remacle et al., 2006; Bonnefoy et al., 2007; Zhou et al., 2010). A mitochondrial targeted adeno-associated virus has also recently proved to be effective as a DNA delivery method in a mouse model system (Yu et al., 2012). All other mitochondrial genetic transformation has

occurred in isolated mitochondria. Two effective plant isolated mitochondrial transfection methods have been developed. The first involves electroporation of wheat mitochondria to study mitochondrial transcription and RNA maturation processes (Farré and Araya, 2001). The second relies upon the natural competence of mitochondria to incorporate exogenous DNA via direct DNA uptake (Koulintchenko et al., 2003). It was discovered that like their evolutionary ancestors  $\alpha$ -proteobacterium, isolated mitochondria maintain a natural competence to import double stranded linear DNA (Leon et al., 1989).

Several critical factors have since been identified as being involved in plant mitochondrial transfection. Mitochondrial import of DNA may involve the voltage-dependant anion channel (VDAC) and the adenine nucleotide translocator (ANT) (Koulintchenko et al., 2003). DNA imported into isolated mitochondria is sequence-independent. The imported DNA is then integrated into the mitochondrial genome through homologous recombination (Leon et al., 1989; Ibrahim et al., 2011; Mileschina et al., 2011). Following recombination into the mitochondrial genome, the exogenous DNA can be transcribed and processed into mature RNA molecules if the gene is controlled by a mitochondrial promoter (Placido et al., 2005; Mileschina et al., 2011). Despite the progress that has been made, no *in vivo* plant mitochondrial transfection method has been developed until now. To do so we tested the hypothesis that a peptide derived from a protein sorting signal and having similar physicochemical properties as a CPP could carry cargo across the outer plasma membrane into the mitochondria of plant cells.

Protein sorting signals are distinct peptide sequences that are referred to as presequences, mitochondrial or mTP sequences for mitochondrial proteins that are transcribed from the nuclear genome and translated in the cytosol. These mitochondrial targeted proteins are recognized by the translocase of the outer/inner membrane

(TOM/TIM) mitochondrial translocation machinery which facilitate the transport of these nascent cytosolic proteins across the mitochondria's double membrane (Neupert and Herrmann, 2007). Peptides that have been identified as having both cell penetrating and specific organelle addressing characteristics are the Szeto-Schiller (SS) peptides that have cell penetrating and mitochondrial targeting properties (Zhao et al., 2004). These antioxidant peptides have a structural motif of alternating aromatic and basic amino acids. They were found to accumulate in the inner mitochondrial membrane due to their combined cationic (3+ charge) and lipophilic properties (Zhao et al., 2004).

Horton et al., (2008) further refined the physicochemical properties needed to target peptides to the mitochondria; peptides require a net positive charge of 3 and a log P value of approximately -2.0 or higher (Horton et al., 2008). Therefore to be targeted to the mitochondria a peptide must have a positive charge and be lipophilic. These structural and chemical features were applied to the development of synthetic mitochondrial penetrating peptides (MPPs) that were tested in 3 human cell lines (Horton et al., 2008). Utilizing the previously identified physicochemical properties of these peptides I applied these criteria to the development of plant organelle targeting cell penetrating peptides.

In this report we describe the development of the first *in vivo* plant cell mitochondrial transfection method. Our method involves the development of short peptides that form nanoparticles composed of peptides and exogenous double-stranded DNA (dsDNA). The DNA is targeted to the mitochondria of cultured plant cells due to the specific properties of the peptides. These short peptides have cell penetrating properties and they have specific organelle targeting properties which enable the peptides to translocate their cargo across the outer plasma membrane of the cell and deliver the dsDNA cargo to the mitochondria. This report describes how the peptides were selected,

screened and utilized to deliver a linear double stranded DNA reporter cassette to the mitochondria and have it expressed *in organello*. Moreover, it demonstrates that germline plant cells with exogenous DNA integrated into the mitochondrial genome can be regenerated into mature plants.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 IDENTIFICATION OF MITOCHONDRIAL TARGETING PEPTIDE SEQUENCES**

Available protein sequences for wheat, rice, maize, and *Arabidopsis* were downloaded from NCBI (National Center for Biotechnology Information) GenBank (09, 2008). To eliminate sequence redundancy within the protein sequence datasets, the computer software program Cluster Database at High Identity with Tolerance (CD-HIT) was used to generate a set of non-redundant sequences (Huang et al., 2010). The protein sequences were then analyzed using the TargetP software to identify N-terminal protein sorting signal sequences specifically, mTP sequences as well as predict the subcellular localization properties of these predicted N-terminal protein sorting signal sequences (Emanuelsson et al., 2000). TargetP uses an algorithm that incorporates a neural network to calculate a transit peptide score and weighted matrices to locate the transit peptide cleavage site. The mTP sequence dataset was parsed according to specific selection criteria to identify mTP sequences with similar physicochemical properties (lipophilic and a net positive charge due to a high concentration of basic amino acids) as cell penetrating peptide. To be selected as a candidate sequence the sequence had to have a TargetP

relative confidence value of  $\geq 0.9$ , a sequence length of  $\leq 35$  amino acids and an average hydrophilicity  $\leq 0$ .

### **3.2.2 PEPTIDE SYNTHESIS AND LABELING**

Thirty one candidate mTP sequences with the highest values selected from those meeting all the criteria outlined above were synthesized using solid phase fluorenylmethoxycarbonyl (Fmoc) chemistry by CanPeptide Inc. Canada. Refer to (Table 3.1) for a full list of the synthesized peptides. Each peptide was labelled with fluorescein isothiocyanate (FITC) at the N-terminus, to facilitate visual detection by fluorescence.

### **3.2.3 MESOPHYLL PROTOPLAST ISOLATION AND PURIFICATION**

Embryonal halves of triticale (cv. AC Ultima) seeds were surface sterilized with 4% hypochlorite for 1 minute and rinsed three times with sterile water. The embryonal halves were then inoculated on basal Murashige and Skoog (MS) media, pH 5.82 (Murashige and Skoog, 1962). Peeled leaves from six-day-old seedlings were incubated in enzyme solution (2% cellulase and 2% macerozyme, Yakult Honsha Co Ltd, Japan) in cell protoplast washing (CPW) solution, pH 5.6 (Frearson, et al. 1973) for 4 hours at 25°C, in the dark. Protoplasts were isolated by centrifugation at 100 g for 3 minutes, at room temperature (Eppendorf centrifuge 5810R, USA), washed twice with CPW solution and purified by layering on 21% sucrose in CPW solution. A band of protoplasts formed at the interphase and was carefully removed and suspended in CPW solution. After two washings with CPW solution, protoplast density was adjusted to  $2 \times 10^5$  protoplasts/ml.

**Table 3.1. Candidate mitochondrial targeting cell penetrating peptides.**

The table describes peptide name, overall charge, hydrophilic value, peptide amino acid sequence and the species from which it was derived.

mTP	Peptide Charge	Hydro-philicity	# aa	Target P Confidence Level	Peptide sequence	Species	GenInfo Identifier	Genbank Accession #
mTP1	7	-0.1	34	0.95	MFSYLPRYPLRAASARALVRATRPSYRSALLRYQ	Wheat	GI:111062043	gii111062043 gb EAT8
mTP2	4	-0.5	20	0.93	MAAWMRSLSPLKKLWIRMH	Maize	GI:195622876	gii195622876 gb ACG3
mTP3	4	-0.4	13	0.92	MKLLWRLILSRKW	Arabidopsis	GI:186525415	gii186525415 ref NP_
mTP4	4	0	14	0.91	MVWRRSRTNSLRYT	Arabidopsis	GI:59798580	gii59798580 sp Q7UHE
mTP5	5	0	16	0.92	MLFRLRRSVRLRGLLA	Arabidopsis	GI:116621918	gii116621918 ref YP_
mTP6	4	-0.4	28	0.93	MASALRLTCAFRAAAPLRTAFNGLRFY	Wheat	GI:111070271	gii111070271 gb EAT9
mTP7	4	-0.3	23	0.91	MSLPRWSQFLRPVFQTSPRVFRAY	Wheat	GI:160702799	gii160702799 gb EAT8
mTP8	5	-0.2	28	0.93	MASLRLRAAQRLPLQTSVRMFSTIPLRY	Wheat	GI:111062572	gii111062572 gb EAT8
mTP9	6	-0.2	34	0.96	MLPRAGLRALRIRPALAVPRITPIASASQWTRTY	Wheat	GI:111070818	gii111070818 gb EAT9
mTP10	6	-0.2	32	0.91	MLQRARLWPIFTSRCAFPFSSRSISIPYRRM	Wheat	GI:160703600	gii160703600 gb EAT8
mTP11	6	-0.1	24	0.97	MRRQRILRRLTFYLLKLDYVVVWRC	Wheat	GI:111061146	gii111061146 gb EAT8
mTP12	4	0	26	0.93	MAVDPRVLRCGAARTIVPPSHHHHRYH	Wheat	GI:111070734	gii111070734 gb EAT9
mTP13	5	0	18	0.92	MFVRTLRTSRAIRAPVRF	Wheat	GI:160703022	gii160703022 gb EAT8
mTP14	4	-0.6	19	0.91	MSLYCGIACRRKFFWCYRL	Rice	GI:51701672	gii51701672 sp Q86X6
mTP15	7	-0.2	35	0.91	MRTKYQYKIRLVTVFFMSRRLTSPRPSSSTVAH	Rice	GI:50726503	gii50726503 dbj BAD3
mTP16	4	-0.1	14	0.96	MFGKRIYRLAIYQR	Rice	GI:182627646	gii182627646 sp Q5VY
mTP17	4	0	14	0.95	MVRRQVCALLRHR	Rice	GI:38346873	gii38346873 emb CAE0
mTP18	5	0	26	0.91	MATAARRLLPALRKSIPAANGAARG	Rice	GI:125560233	gii125560233 gb EAZ0
mTP19	4	-0.3	19	0.92	MLLLSSNIRRRWLTCPCRL	Maize	GI:194692306	gii194692306 gb ACF8
mTP20	4	-0.2	17	0.95	MLRGFRLHRCPYWRPS	Maize	GI:195654757	gii195654757 gb ACG4
mTP21	5	-0.1	17	0.95	MAAWWRRKVVPCCARRAW	Maize	GI:195629372	gii195629372 gb ACG3
mTP22	4	0	14	0.95	MATTRLLPLRRLR	Maize	GI:195639640	gii195639640 gb ACG3
mTP23	6	0	22	0.92	MKCLGRYAIKLLSRRLPSIRF	Arabidopsis	GI:186478941	gii186478941 ref NP_
mTP24	9	0	28	0.98	MMARLFWIRARLRLIRRHPRHLAFRT	Arabidopsis	GI:92115761	gii92115761 ref YP_5
mTP25	4	-0.5	21	0.95	MGWLRHLHSLFSLRRMWVRAH	Maize	GI:195646572	gii195646572 gb ACG4
mTP26	8	-0.2	31	0.96	MRRILYRRFSNRAIFGKFIKVLHSLRCL	Arabidopsis	GI:89257674	gii89257674 gb ABD65
mTP27	5	-0.3	35	0.90	MAMAMMAMRRAVALGARHIPAAAASSFRVVSLRHM	Maize	GI:194706144	gii194706144 gb ACF8
mTP28	6	-0.4	33	0.90	MQWRGRFRYPLLRSLIAIRPLSPILLALCCRL	Rice	GI:125544178	gii125544178 gb EAY9
mTP29	7	-0.1	34	0.94	MAASRLLRPAARLAFRPAFPAFPAIVARRCY	Wheat	GI:111063598	gii111063598 gb EAT8
mTP30	5	-0.3	18	0.94	MPPKPMWRFWLWFGKRR	Wheat	GI:111066796	gii111066796 gb EAT8
mTP31	4	-0.4	30	0.93	MLLYGGLAVAGWRARRVGAARPLVASVPAV	Wheat	GI:124386035	gii124386035 ref YP_

### **3.2.4 MICROSPORE ISOLATION AND PURIFICATION**

Triticale (cv. Ultima) microspores at the mid-late uninucleate stage were isolated from surface sterilized anthers carried out in NPB-99 medium, pH 7.0 (Eudes and Amundsen, 2005).

### **3.2.5 INCUBATION OF ISOLATED TRITICALE PROTOPLASTS OR MICROSPORES WITH FLUORESCENTLY LABELLED PEPTIDES**

Mesophyll protoplasts or microspores (500 µl of  $2 \times 10^5$  cells/ml preparations) were incubated with 180 µl fluorescently labelled mTPs (100 µM) for 1 hour in the dark at room temperature, followed by washing with CPW solution for protoplasts or NPB-99 media for microspores. The isolated cells were then treated with trypsin-ethylenediaminetetraacetic acid (0.25%, Sigma-Aldrich) in CPW solution for protoplasts or in NPB-99 media for microspores (1:4) for 5 minutes followed by washing with CPW solution for protoplasts or NPB-99 media for microspores. Finally the cells were suspended in CPW solution (500 µl) for protoplasts or NPB-99 media (500 µl) for microspores.

### **3.2.6 CONFOCAL MICROSCOPY**

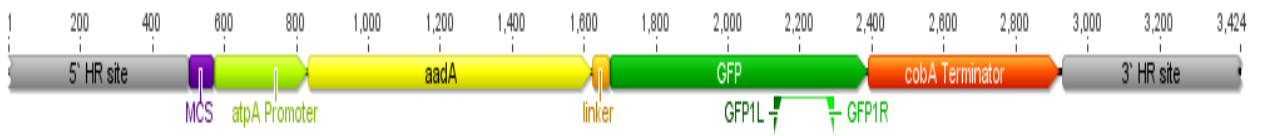
Cells were observed using a confocal microscope (Nikon C1, Nikon Canada Inc.) to analyze the subcellular localization of the fluorescently labelled mTPs (excitation wavelength 490 nm / emission wavelength 520 nm). The fluorescent dye MitoTracker<sup>®</sup> Orange CM-H<sub>2</sub>TMRos (M7511, Invitrogen) was used to stain mitochondria according to the manufactures protocol (excitation wavelength 554 nm / emission wavelength 576 nm). Fluorescence emissions were collected in z-confocal planes of 10–15 nm and

analyzed using EZ-C1 Software Version 3.6. Three-dimensional images were acquired using the following settings on Nikon EZ-C1 software (Gold version 2.3; Image Systems Inc., Columbia, MD): Nikon plan apoc-hromatic 100 X A oil objective (1.49 numerical aperture), 3.6- $\mu$ s scan dwell time, 512 X 512 pixel size resolution, 0.30  $\mu$ m z-step, and a 150- $\mu$ m detector pinhole. The images were analyzed for 3D colocalized fluorescence between the fluorescently labelled peptides and MitoTracker<sup>®</sup> stained mitochondria.

### **3.2.7 REPORTER GENE CONSTRUCT FOR EXPRESSION IN MITOCHONDRIA**

Wheat mitochondrial *aadA:gfp* reporter cassette is a 3424 base pair wheat specific mitochondrial transfection vector, designed to target insertions in between the *trnfM-1* and *rrn18-1* gene cluster which is repeated three times in the *Triticum aestivum* mitochondrial genome (Fig. 3.1). The vector was designed using Vector NTI<sup>®</sup> and synthesised by GeneArt<sup>®</sup>. The nucleotide sequences 300376-300875 and 300877-301370 (GenBank accession No. AP008982.1) were used as the left and right sequences flanking the cassette to be introduced by homologous recombination. These sequences share significant amounts of homology with the other two *trnfM* and *rrn18* repeat regions. A multiple cloning site was introduced following the *trnfM* target insertion sequence. The selection marker gene was the *aadA:gfp* fusion gene, which facilitates a dual selection method of spectinomycin / streptomycin antibiotic resistance through an *aadA* derivative and visual detection by green fluorescent protein (GFP) fluorescence (GenBank accession No. ABX39486) (Khan and Maliga, 1999). The selection marker gene was driven by the *Triticum aestivum* mitochondrial *atpA* gene promoter (GenBank accession No.X54387.1). The *aadA:gfp* fusion gene was terminated with the TcobA terminator sequence derived





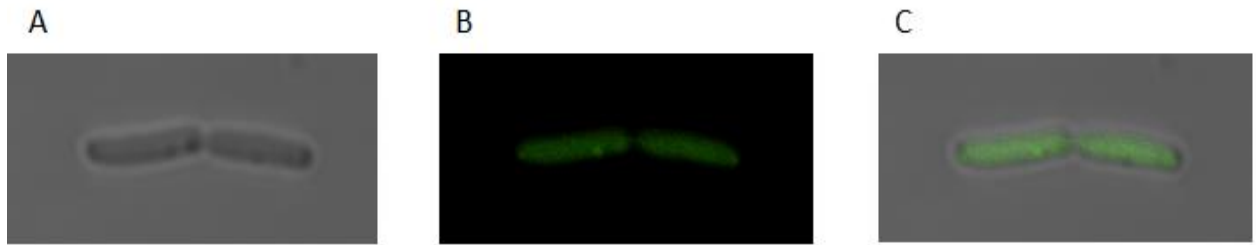
**Fig. 3.1: A map of the *aadA:gfp* reporter cassette.**

5' HR site = homologous recombination sequence from wheat mitochondrial *trnfM18-1* gene. MCS = multiple cloning site. *atpA* Promoter = Wheat mitochondrial *atpA* promoter. *aadA* = aminoglycoside-3'-adenyltransferase spectinomycin / streptomycin resistance gene. Linker = 16 amino acid linker between the *aadA* and *gfp* genes. GFP = green fluorescent protein (*gfp*) gene. *cobA* Terminator = Rice *cobA* terminator. 3' HR site = homologous recombination sequence from wheat mitochondrial *rrn18-1* gene. GFP1L and GFP1R = GFP primer binding sites and amplicon representation.

from the *Triticum aestivum* mitochondrial genome at nucleotides 62871-62565 (GenBank accession No. AP008982.1). To test that the construct produced the aadA:gfp protein the 6983 base pair wheat mitochondria *aadA:gfp* reporter plasmid was transformed into *E.coli* Rosetta cells according to Novagen<sup>®</sup> protocol. PCR was used to confirm the plasmid was present within the cells. The cells were also observed using confocal microscopy to confirm GFP expression (Fig. 3.2).

### 3.2.8 GEL MOBILITY SHIFT ASSAY

The gel mobility shift assay was used to determine the minimum peptide concentration needed to bind to linearized plasmid DNA and cause it to shift during electrophoresis. Purified linearized plasmid DNA (100 ng of linear double stranded DNA, 6.8 kb) was mixed with increasing concentrations of each of the first five mTPs 1 to 5 listed in (Table. 3.1), according to calculated peptide:DNA charge ratios (charge of peptide / charge of DNA 0:1, 1:1, 2:1, 3:1, 4:1, 5:1, to a maximum of 2050:1). To perform the peptide:DNA charge ratio calculation we considered that 1  $\mu$ g of DNA contains 3.03 nM of phosphate and therefore has -3 nM of charge; based on the charge and molecular weight of each peptide it was calculated how much peptide would be required to neutralize the DNAs charge on an integer basis. A reaction containing only plasmid DNA was included as a negative control, which is indicated by the ratio 0:1. The DNA was prepared to a final concentration of a 100 ng/ $\mu$ l in sterile water. Each reaction had a final volume of 25  $\mu$ l and was incubated for 30 minute, and then subjected to electrophoresis on 1% agarose gel stained with ethidium bromide (Sambrook and Russell, 2006). This procedure was repeated until a complete shift was observed in the plasmid



**Fig. 3.2: Confocal images of *E. coli* Rosetta cells transformed with wheat mitochondrial *aadA:gfp* reporter plasmid.**

A) Differential interference contrast (DIC) channel showing transformed *E. coli* Rosetta cells. B) Green channel showing transformed *E. coli* Rosetta cells. C) A composite image of the DIC (*E. coli* Rosetta cells) and green (GFP) channels.

DNA or a maximum peptide:DNA charge ratios of 2600:1 was reached. A maximum peptide:DNA charge ratios of 2600:1 was chosen because higher concentrations of peptide would translate into requiring too much peptide in the transfection reaction and would have made it financially prohibitive.

### **3.2.9 NUCLEASE PROTECTION ASSAY**

The first five mTPs 1 to 5 listed in Table 3.1 were separately mixed with linearized plasmid DNA as described for the gel mobility shift assay. For the nuclease protection assay, 5  $\mu$ l of DNase I (RNase-free DNase set; Qiagen, Valencia, CA, USA) was added to the mixture volume (50  $\mu$ l). The mixture was incubated at room temperature for 15 minutes and then incubated on ice for 5 minutes. Plasmid-peptide dissociation and plasmid purification was carried out with a commercially available DNA purification kit (QIAquick™ PCR purification kit; Qiagen). DNA was eluted in sterile water. An aliquot of 6  $\mu$ l was subjected to 1% agarose gel electrophoresis.

### **3.2.10 PROTOPLAST AND MICROSPORE TRANSFECTION WITH *aadA:gfp* REPORTER**

The linearized dsDNA construct 1.5  $\mu$ g was combined with one of each of the 5 mTPs (scaled up from 100 ng, at 4 times the concentration needed to cause a DNA shift in the gel mobility shift assay) in a final volume of 100  $\mu$ l in CPW solution for protoplasts or NPB-99 media for microspores. The mixture was incubated for 10 minutes at room temperature, and then incubated with isolated triticale mesophyll protoplasts (500  $\mu$ l of  $2 \times 10^5$  protoplasts/ml) for 1 hour in the dark at room temperature. CPW solution for protoplasts or NPB-99 media for microspores (500  $\mu$ l) was added and the mixture was

incubated in the dark for 24 hours. The cells were imaged using confocal microscopy, using MitoTracker<sup>®</sup> Orange CM-H<sub>2</sub>TMRos (Molecular Probes<sup>®</sup>) staining to visualize mitochondria according to the manufacturer's protocol.

### **3.2.11 DNA AND RNA EXTRACTION OF TRANSFECTED PROTOPLASTS, MICROSPORES AND PLANT TISSUE**

Following a 48 hours, 21°C incubation, transfected protoplasts, microspores or regenerated plant tissue were frozen at -80°C. These frozen samples were disrupted using ceramic beads and aggressive shaking for 1 minute in a 2.0 ml microcentrifuge tube. An aliquot of 450 µl of RLT buffer/B-Me from Qiagen's All Prep DNA/RNA mini kit was added to the sample and then it was vortexed for 10 seconds. The samples were heated at 55°C for 1 minute and vortexed for 10 seconds again. Extraction of the DNA and RNA was performed using Qiagen's All Prep DNA/RNA mini kit using the on column DNase I digestion according to the manufacturer's protocol. The final elution volume was 40 µl. DNA and RNA quantity and quality was assessed using an agarose gel (to ensure DNA and RNA integrity) and quantified spectrophotometrically.

### **3.2.12 REVERSE TRANSCRIPTION PCR (RT-PCR)**

cDNA was generated using a SuperScript<sup>®</sup> III First-Strand Synthesis Kit (Life Technologies). One µg of RNA was added to each reaction and the manufacturer's protocol for synthesis using random priming was followed, with the exception that the volume of the reaction mixture was increased from 20 µl to 30 µl. A negative reverse

transcription reaction containing all reagents but no RNA was included in the cDNA synthesis as a control.

All real time PCR reactions were performed using QuantiTect™ SYBR™ Green PCR Master Mix (Qiagen) in a 20 µl reaction volume. Each reaction contained 1 µM of each primer GFP4L 5'-acatcacggcagacaaacaa-3' and GFP4R 5'-aaagggcagattgtgtggac-3' and was tested in triplicate using 3 µl of template in each reaction. Cycling was performed as follows: 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds. Dissociation curves were run after the PCR reactions were complete. The 3 CT values were averaged for each sample and a standard deviation was calculated. Standard curves were generated by plotting average CT value against the log of the amount of DNA in each sample so that the PCR efficiency of each primer set could be assessed. The primer sets were amplified with comparable efficiency so CT values could be directly compared.

Standard curves were generated for both elongation factor 1 alpha (*EF1α*) (housekeeping control) and *aadA:gfp* genes. The *EF1α* curve was generated by pooling 6 µl of each cDNA sample (excluding the negative reverse transcription control), then carrying out a two fold serial dilution five times. The GFP standard curve was generated using the plasmid DNA used for transfection (13 ng/µl plasmid, of which 3 µl (40 ng) were used for the PCR reaction, followed by a two fold serial dilution five times).

Relative expression levels of *aadAGFP /EF1α* were measured using the outlined RT-PCR method. The mean relative expression levels of three protoplast transfections performed using each of the five cTP peptides delivering the *aadAGFP* reporter cassette were analyzed by one-way ANOVA using SPSS 15.0 software. P values ( $P \leq 0.05$ ) were utilized to determine if the measured changes in transcript levels were significant.

### **3.2.13 MICROSPORE TRANSFECTION WITH REPORTER CASSETTE AND PLANT PROPAGATION**

To test the application of mitochondrial transfection in the microspore tissue culture system using mTP1, we applied the previously described microspore transfection protocol to genetically engineer the mitochondrial genome of triticale microspores. Following the 1 hour incubation after the microspore transfection, 100 mg/L of streptomycin was added to the induction media, and cultured in the dark at 27°C. At 4 weeks developing embryos were transferred to a Rita Box semi-immersion culture system using 200 ml of liquid GEM supplemented with 200 µl PPM™, beneath Sylvania GRO-LUX® wide spectrum bulbs (40 watts) delivering 80 µM m<sup>-2</sup> s<sup>-1</sup> (16 hour light period) with a room temperature at 16°C. The following streptomycin selection regime was applied to the developing embryos (week 1 - 4, 100 mg/L, week 5, 200 mg/L and week 7, 400 mg/L streptomycin selection). At 9 weeks, germinated (green and variegated) plantlets were transferred to soil, in root trainers. The streptomycin selective pressure was not maintained in soil. The microspore control cultures were carried in absence of transfecting peptide or DNA. Two sets of microspore control cultures were prepared, 1 set containing streptomycin and the other without to determine the effect antibiotics would have on green plant regeneration.

### **3.2.14 MOLECULAR EVALUATION OF T<sub>0</sub> PLANTLETS**

Genomic DNA was isolated from 5 cm young leaves of the primary transformants (T<sub>0</sub>) at the end of the *in vitro* culture steps, using DNeasy Plant Mini Kit (Qiagen). To determine which plants were positive for the reporter, the PCR was performed with 2 µl

of template in a final volume of 20  $\mu$ l. Each reaction contained 1  $\mu$ M of each primer GFP1L 5'-cacggcagacaacaaaaga-3' and GFP1R 5'-aaagggcagattgtgtggac-3' (Fig. 3.1). Cycling was performed as follows: 94°C for 3 minutes, 30 repeats of 94°C for 1 minute, 60°C for 30 seconds, 72°C for 30 seconds, final extension of 72°C for 10 minutes. Samples were separated by electrophoresis on a 1.0% agarose gel in 1 $\times$  TAE buffer for 1 hour.

### 3.2.15 SEQUENCING

PCR amplified genomic DNA was used to confirm the integration of the *aadA:gfp* gene into the three target sites within the mitochondrial genome. An alignment of the nucleotide sequences from all three of the repeat regions 18-1 (294591 – 312591), 18-2 (385976 – 403976) and 18-3 (49881 – 67881) (GenBank accession No. AP008982.1) was made using Geneious (version 6.1.3, Biomatters). The consensus sequence was generated by extending the left and right 500 bp homologous recombination regions of the reporter cassette by an additional 500 bp flanking mitochondrial genomic sequence. The extended reporter cassette was used to develop gene targeting specific PCR primers for (rrn18-1) trnfMF3 5'-tttcatccactcctcatcc-3', trnfMR2 5'-gatcaccgcttcctcat-3', rrn18-1F3 5'-gtccacacaatctgcccttt-3', rrn18-1R4 5'-cctttgagtttcggttgc-3', (rrn18-2) trnfMF6 5'-gctgcctactcatccaag-3', trnfMR4 5'-tcaccgtaaccagcaaatca-3', rrn18-2F5 5'-gtccacacaatctgcccttt-3', rrn18-2R6 5'-ttgcgcggttatcgaatta-3' and (rrn18-3) trnfMF5 5'-ccctcaattggtcatgctt-3', trnfMR4 5'-tcaccgtaaccagcaaatca-3', rrn18-3F4 5'-tcacggcagacaacaaaag-3', rrn18-3R5 5'-aaggttttcgcggttgc-3'. The PCR products were gel extracted and purified using a Gel Extraction Kit (Qiagen). The PCR fragments were cloned into a pCR® 2.1 Vector TA Cloning® Kit (Invitrogen) for DNA sequencing. The



cloned PCR products were sequenced by McGill University and Genome Quebec Innovation Center (Montreal, QC).

### **3.3 RESULTS**

#### **3.3.1 mTP SELECTION AND EVALUATION**

The total number of Genbank plant protein sequences downloaded 785 615 was reduced to 748, when TargetP predicted mTP sequences were parsed sequentially and cumulatively using the selection criteria of TargetP relative confidence value of  $\geq 0.9$ , a sequence length of  $\leq 35$  amino acids and an average hydrophilicity  $\leq 0$ . Thirty one of the 748 TargetP predicted mTP sequences, defined as having a charge ratio of above 3.5 and a hydrophilicity of  $\leq 0$ , were selected and synthesized (Table 3.1). Each peptide was labelled with FITC at the N-terminus to facilitate visual detection by fluorescence and then tested for cell penetrating and mitochondrial targeting properties using confocal microscopy in a co-localization assay to make these observations.

There was a large range of ability to transduce across the outer plasma membrane and specifically target the mitochondria among the tested peptides. Numerous peptides failed to cross the outer plasma membrane and appeared as large and small patches of agglutinated fluorescent peptide on the outer surface of the protoplasts (data not shown). These peptides were deemed to not have any cell penetrating properties. Other peptides did cross the outer plasma membrane of the protoplasts, therefore did possess cell penetrating properties, but didn't specifically target the mitochondria and did not fulfill the second criteria of specific organelle targeting (data not shown). Six peptides exhibited weak diffuse fluorescents within the cytosol, four peptides were observed to be contained within endosomes, four peptides exhibited nonspecific localization around the surface of

the chloroplasts and eleven were observed to be contained within endosomes and aggregate at the chloroplast surface (data not shown). Out of the thirty one peptides tested, five mTPs were found to both penetrate the outer plasma membrane and specifically localize to the mitochondria (Fig. 3.3).

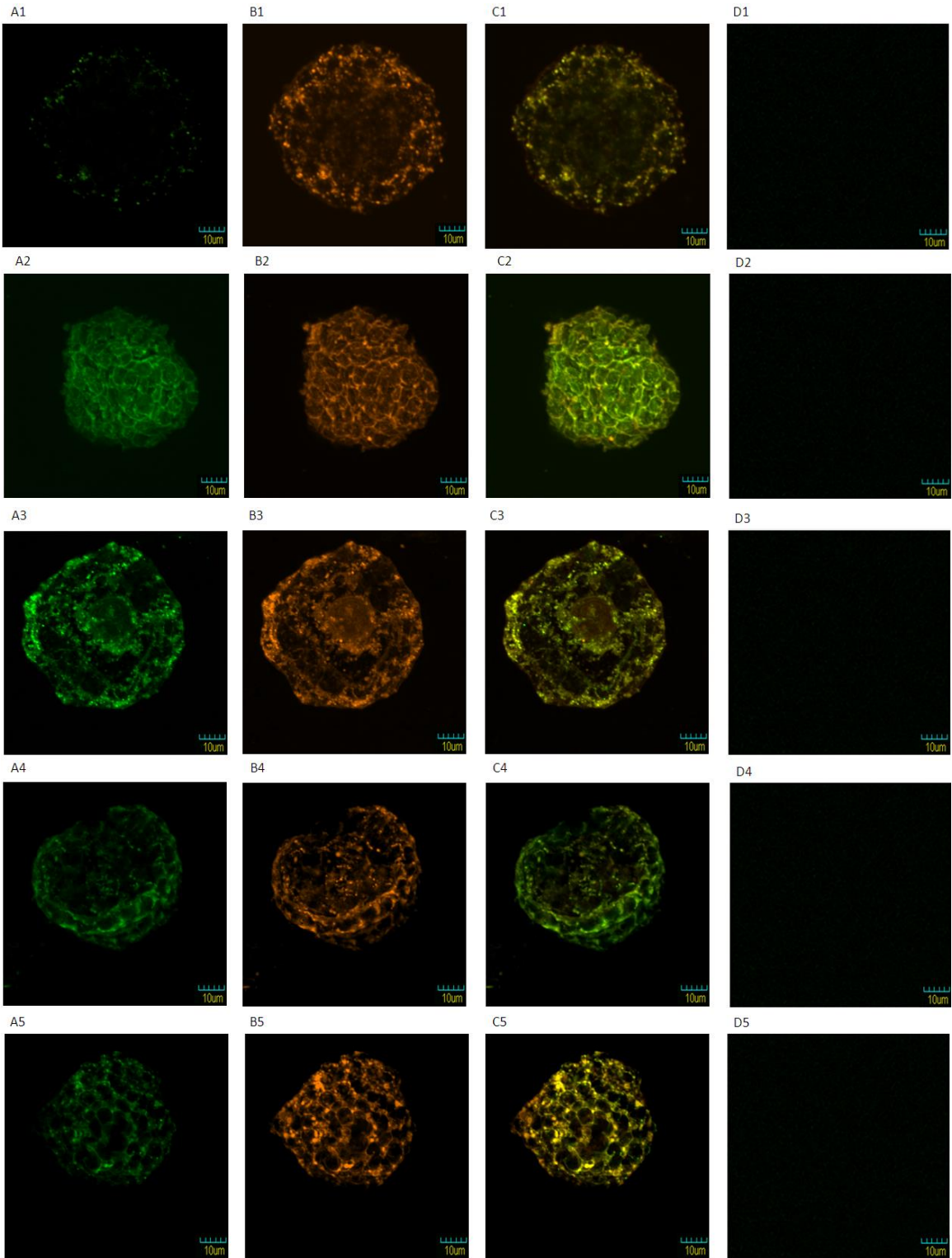
The five mTPs, having both cell penetrating and mitochondrial targeting properties in protoplasts (mTP1-mTP5, Table 3.1), were also tested for these properties in isolated triticale microspores. It was determined that mTPs accumulate within microspores due to the large fluorescent signals observed over the corrected background fluorescent levels (data not shown). This indicates that the mTPs have the ability to translocate across the plasma membrane of isolated microspores surrounded by exine and intine. The fluorescent dye MitoTracker<sup>®</sup> Orange CM-H<sub>2</sub>TMRos did not specifically stain the mitochondria of microspores therefore we could not conclude exactly where the mTPs were specifically localizing in the transfected microspores.

### **3.3.2 mTP DNA BINDING AND PROTECTION ANALYSIS**

To determine if the five mTPs having both cell penetrating and mitochondrial targeting properties could be utilized to deliver cargo into cells, the peptides were tested for their ability to non-covalently bind to nucleic acids. A gel mobility shift assay and a nuclease protection assay were separately performed with each peptide to determine the minimum peptide concentration needed to bind to and completely saturate the linearized dsDNA in preparation for the protoplast and microspore transfections. The results of these assays are shown in (Table 3.3 and Fig. 3.4 – 3.7). Four out of the five mTPs (mTP1, mTP2, mTP3 and mTP5) caused a mobility shift in the DNA during

**Fig. 3.3: Confocal images of a FITC labelled mTPs 1-5 transfected protoplasts.**

A1-5) Green channel showing the FITC labelled mTP1-5 peptide has transduced across the outer plasma membrane of the transfected protoplast. B1-5) Orange channel showing the MitoTracker® Orange CM-H2 TMRos stained mitochondria. C1-5) A composite image of the orange (mitochondria) and green (peptide) channels. This image shows the co-localization of the green fluorescently labelled peptide with the orange stained mitochondria which appears yellow in the image. The yellow intensity varies according to the green/orange ratio or co-localization of the fluorescently labelled peptide and MitoTracker® stained mitochondria within the image. D1) Negative control image of protoplasts with the background subtracted green and red channels.



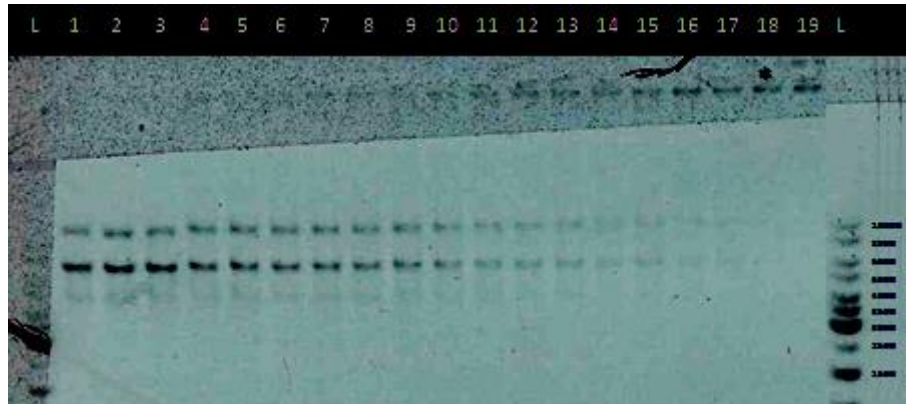
**Table 3.2. mTP sequence selection criteria.**

The first 3 columns of numerical data represent the total number of starting protein sequences from NCBI GenBank from each organism, the total number of mTP sequences predicted by TargetP and the predicted number of mTP sequences with a TargetP relative confidence level of  $\geq 90\%$ , respectively. The remaining columns of numerical data represent the numbers of sequences predicted when the following selection criteria were applied sequentially and cumulatively: a sequence length of 35 amino acids or less, a net positive charge of  $\geq 3.5$  and an average hydrophilicity of  $\leq 0$ .

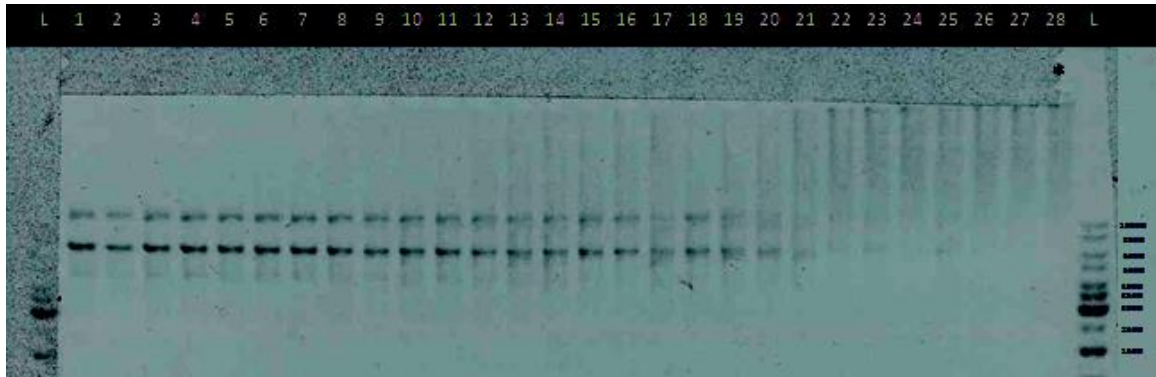
Species	Total starting protein sequences	mTP Sequences	Relative Confidence Level $\geq 0.9$	Sequence Length $\leq 35$	Net Positive Charge $\geq 3.5$	Average Hydrophilicity $\leq 0$
<i>Arabidopsis</i>	387 781	45 588	1 619	503	488	282
Rice	302 221	55 065	4 635	1 167	701	362
Maize	60 709	10 251	423	250	139	81
Wheat	34 904	4 594	312	81	23	23
Totals	785 615	115 498	6 989	2 001	1 351	748

**Table 3.3 DNA binding properties of mitochondrial targeting cell penetrating peptides.**

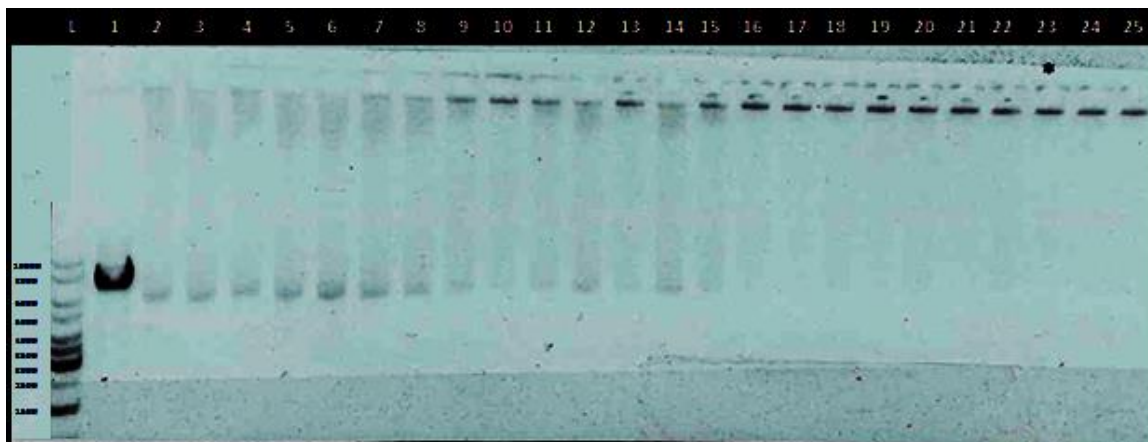
<b>Peptide</b>	<b>Peptide : DNA Molar Binding Ratio</b>	<b>Nuclease Protection</b>
<b>mTP1</b>	<b>3.8</b>	<b>+</b>
<b>mTP2</b>	<b>6.0</b>	<b>+</b>
<b>mTP3</b>	<b>14.0</b>	<b>+</b>
<b>mTP4</b>	<b>-</b>	<b>+</b>
<b>mTP5</b>	<b>8.8</b>	<b>+</b>



**Fig. 3.4: Gel mobility shift assay of linearized plasmid DNA incubated with mTP1.** The linearized plasmid DNA was packaged with various molar concentrations of peptide that correspond to calculated peptide:DNA charge ratios. L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) 100:1, 2) 125:1, 3) 150:1, 4) 175:1, 5) 200:1, 6) 225:1, 7) 250:1, 8) 275:1, 9) 300:1, 10) 325:1, 11) 350:1, 12) 375:1, 13) 400:1, 14) 425:1, 15) 450:1, 16) 475:1, 17) 500:1, 18) 525:1, 19) 550:1.  
\* Indicates the point where a complete shift was observed in the plasmid DNA. The molar ratio of peptide:DNA was 3.8:1.

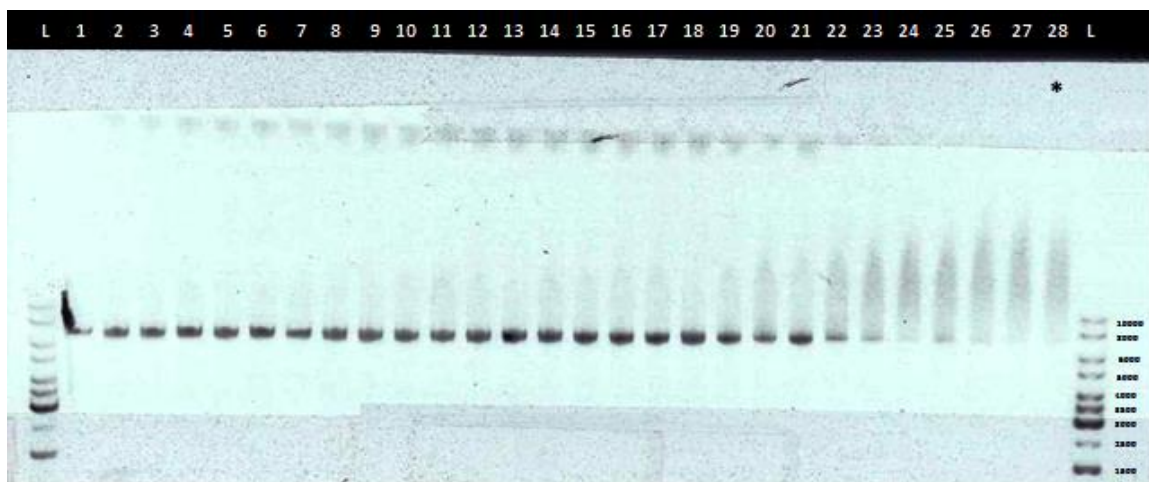


**Fig. 3.5: Gel mobility shift assay of linearized plasmid DNA incubated with mTP2.** The linearized plasmid DNA was packaged with various molar concentrations of peptide that correspond to calculated peptide:DNA charge ratios. L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) 100:1, 2) 125:1, 3) 150:1, 4) 175:1, 5) 200:1, 6) 225:1, 7) 250:1, 8) 275:1, 9) 300:1, 10) 325:1, 11) 350:1, 12) 375:1, 13) 400:1, 14) 425:1, 15) 450:1, 16) 475:1, 17) 500:1, 18) 525:1, 19) 550:1, 20) 575:1, 21) 600:1, 22) 625:1, 23) 650:1, 24) 675:1, 25) 700:1, 26) 725:1, 27) 750:1, 28) 775:1.  
\* Indicates the point where a complete shift was observed in the plasmid DNA. The molar ratio of peptide:DNA was 6.0:1.



**Fig. 3.6: Gel mobility shift assay of linearized plasmid DNA incubated with mTP3.** The linearized plasmid DNA was packaged with various molar concentrations of peptide that correspond to calculated peptide:DNA charge ratios. L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) 1:1, 2) 2025:1, 3) 2050:1, 4) 2075:1, 5) 2100:1, 6) 2125:1, 7) 2150:1, 8) 2175:1, 9) 2200:1, 10) 2225:1, 11) 2250:1, 12) 2275:1, 13) 2300:1, 14) 2325:1, 15) 2350:1, 16) 2375:1, 17) 2400:1, 18) 2425:1, 19) 2450:1, 20) 2475:1, 21) 2500:1, 22) 2525:1, 23) 2550:1, 24) 2575:1, 25) 2600:1.  
 \* Indicates the point where a complete shift was observed in the plasmid DNA. The molar ratio of peptide:DNA was 14.0:1.





**Fig. 3.7: Gel mobility shift assay of linearized plasmid DNA incubated with mTP5.** The linearized plasmid DNA was packaged with various molar concentrations of peptide that correspond to calculated peptide:DNA charge ratios. L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) 1:1, 2) 1125:1, 3) 1150:1, 4) 1175:1, 5) 1175:1, 6) 1200:1, 7) 1225:1, 8) 1250:1, 9) 1275:1, 10) 1300:1, 11) 1325:1, 12) 1350:1, 13) 1375:1, 14) 1400:1, 15) 1425:1, 16) 1450:1, 17) 1475:1, 18) 1500:1, 19) 1525:1, 20) 1550:1, 21) 1575:1, 22) 1600:1, 23) 1625:1, 24) 1650:1, 25) 1675:1, 26) 1700:1, 27) 1725:1, 28) 1750:1.

\* Indicates the point where a complete shift was observed in the plasmid DNA. The molar ratio of peptide:DNA was 8.8:1.

electrophoresis at specific peptide:DNA molar binding ratios. This demonstrated that all but one of the peptides were able to form a stable, non-covalent bond between the negatively charged DNA and the positively charged peptides, in the presence of an electric field.

The peptide:DNA molar binding ratios varied from 3.8 M for mTP1, 6.0 M for mTP2, 14.0 for mTP3 and 8.8 M for mTP5. Variation in amino acid sequence and charge along the peptides may explain why there was variability in terms of the ability of each peptide to bind to the linearized dsDNA and cause a shift during electrophoresis as well as mTP4s inability to cause a mobility shift in the DNA during electrophoresis.

A factor to consider when introducing nucleic acids into the cytosol of a cell is nuclease degradation of the nucleic acids. The nuclease protection assay determined that all of the mTPs were able to protect the DNA from nuclease degradation at or above the peptide:DNA molar ratios, as determined using the gel mobility shift assay (Table 3.3 and Fig. 3.8 – 3.11).

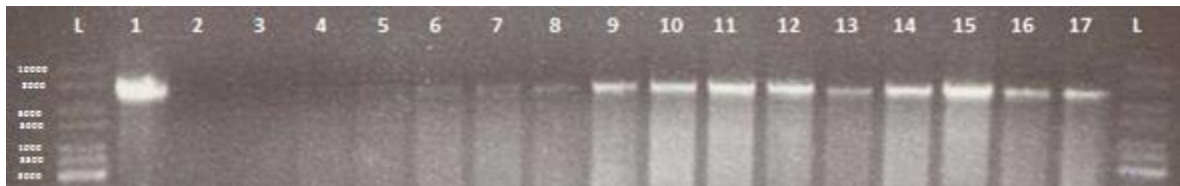
### **3.3.3 mTPs CAN BE USED TO DELIVER A DNA REPORTER CASSETTE TO MITOCHONDRIA IN TISSUE CULTURED PLANT CELLS**

The peptide reporter cassette transfection assay was used to determine if the five candidate peptides could be used to transfect the *aadA:gfp* reporter cassette into triticale mesophyll protoplast or microspore mitochondria. Following transfection, the triticale mesophyll protoplast mitochondria stained with the fluorescent dye MitoTracker<sup>®</sup> Orange CM-H<sub>2</sub>TMRos were observed to fluoresce in both the green and orange background subtracted channels. The co-localization of the two fluorescent signals indicates that the



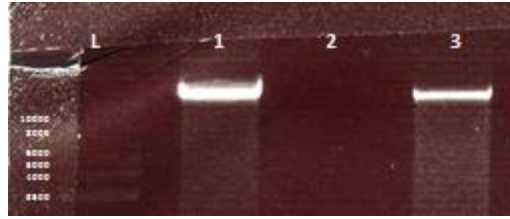
**Fig. 3.8: Nuclease protection of 100 ng of linearized plasmid DNA by varying concentrations of mTP1.**

L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) Linearized plasmid DNA. 2) Linearized plasmid DNA plus DNase I. 3) mTP1plus linearized plasmid DNA at a molar binding ratio 8.3:1 and DNase I. 4) mTP1plus linearized plasmid DNA at a molar binding ratio 16.7:1 and DNase I. 5) mTP1plus linearized plasmid DNA at a molar binding ratio 25.0:1 and DNase I. 6) mTP1plus linearized plasmid DNA at a molar binding ratio 33.3:1 and DNase I. 7) mTP1plus linearized plasmid DNA at a molar binding ratio 45.0:1 and DNase I.



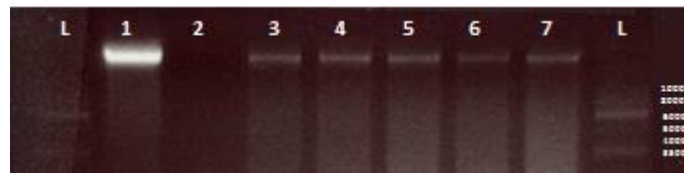
**Fig. 3.9: Nuclease protection of 100 ng of linearized plasmid DNA by varying concentrations of Tat, mTP2 and mTP3.**

L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) Linearized plasmid DNA. 2) Linearized plasmid DNA plus DNase I. 3) Tat plus linearized plasmid DNA at a molar binding ratio 6.8:1 and DNase I. 4) Tat plus linearized plasmid DNA at a molar binding ratio 13.4:1 and DNase I. 5) Tat plus linearized plasmid DNA at a molar binding ratio 20.2:1 and DNase I. 6) Tat plus linearized plasmid DNA at a molar binding ratio 26.9:1 and DNase I. 7) Tat plus linearized plasmid DNA at a molar binding ratio 33.7:1 and DNase I. 8) mTP2 plus linearized plasmid DNA at a molar binding ratio 13.0:1 and DNase I. 9) mTP2 plus linearized plasmid DNA at a molar binding ratio 26.3:1 and DNase I. 10) mTP2 plus linearized plasmid DNA at a molar binding ratio 39.5:1 and DNase I. 11) mTP2 plus linearized plasmid DNA at a molar binding ratio 52.7:1 and DNase I. 12) mTP2 plus linearized plasmid DNA at a molar binding ratio 65.9:1 and DNase I. 13) mTP3 plus linearized plasmid DNA at a molar binding ratio 30.8:1 and DNase I. 14) mTP3 plus linearized plasmid DNA at a molar binding ratio 61.7:1 and DNase I. 15) mTP3 plus linearized plasmid DNA at a molar binding ratio 92.5:1 and DNase I. 16) mTP3 plus linearized plasmid DNA at a molar binding ratio 123.3:1 and DNase I. 17) mTP3 plus linearized plasmid DNA at a molar binding ratio 154.1:1 and DNase I.



**Fig. 3.10: Nuclease protection of 100 ng of linearized plasmid DNA by varying concentrations of mTP4.**

L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) Linearized plasmid DNA. 2) Linearized plasmid DNA plus DNase I. 3) mTP4 plus linearized plasmid DNA at a molar binding ratio 96.7:1 and DNase I.



**Fig. 3.11: Nuclease protection of 100 ng of linearized plasmid DNA by varying concentrations of mTP5.**

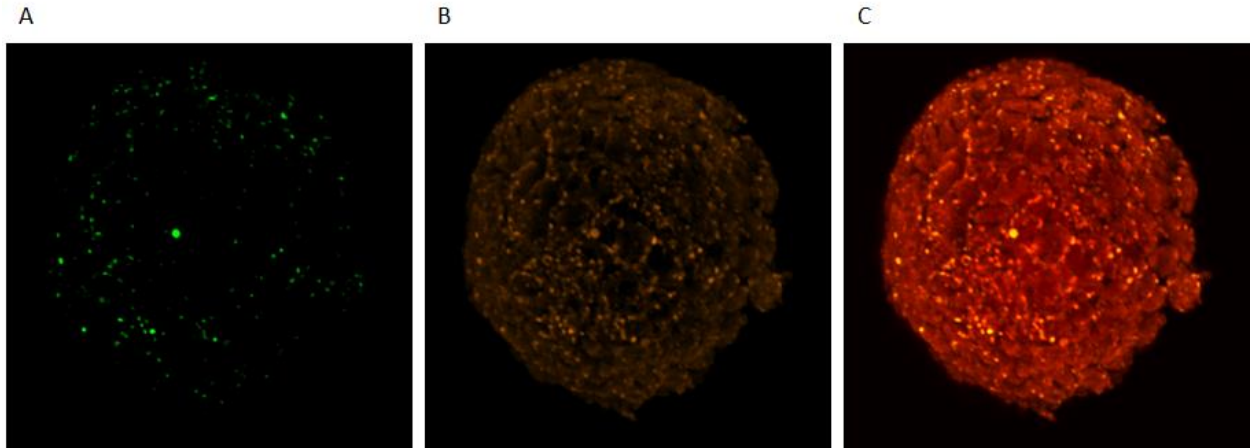
L) Thermo Scientific™ Fermentas™ GeneRuler™ DNA Ladder Mix. 1) Linearized plasmid DNA. 2) Linearized plasmid DNA plus DNase I. 3) mTP5 plus linearized plasmid DNA at a molar binding ratio 19.9:1 and DNase I. 4) mTP5 plus linearized plasmid DNA at a molar binding ratio 38.8:1 and DNase I. 5) mTP5 plus linearized plasmid DNA at a molar binding ratio 58.1:1 and DNase I. 6) mTP5 plus linearized plasmid DNA at a molar binding ratio 77.5:1 and DNase I. 7) mTP5 plus linearized plasmid DNA at a molar binding ratio 96.9:1 and DNase I.

mitochondria in the mTP *aadA:gfp* reporter cassette transfected protoplasts were expressing GFP (Fig. 3.12). Figure 3.12 is a representative image demonstrating that peptide mTP2 can deliver the linear dsDNA reporter cassette to the mitochondria and have it expressed *in organello*. Similar observations were made with the four remaining peptides; however, images of mitochondrial GFP expression in transfected protoplasts were generated for two peptides, mTP2 and mTP4 (Fig. 3.12 and 3.13). Variability in these observations and lack of images for all the peptides can be explained by the fact that only a subset of the entire transfected cell population was used to prepare slides for confocal microscopy and in the technical difficulty of working with triticale protoplasts.

### **3.3.4 EXPRESSION ANALYSIS OF mTP *aadA:gfp* CASSETTE TRANSFECTED PLANT CELLS**

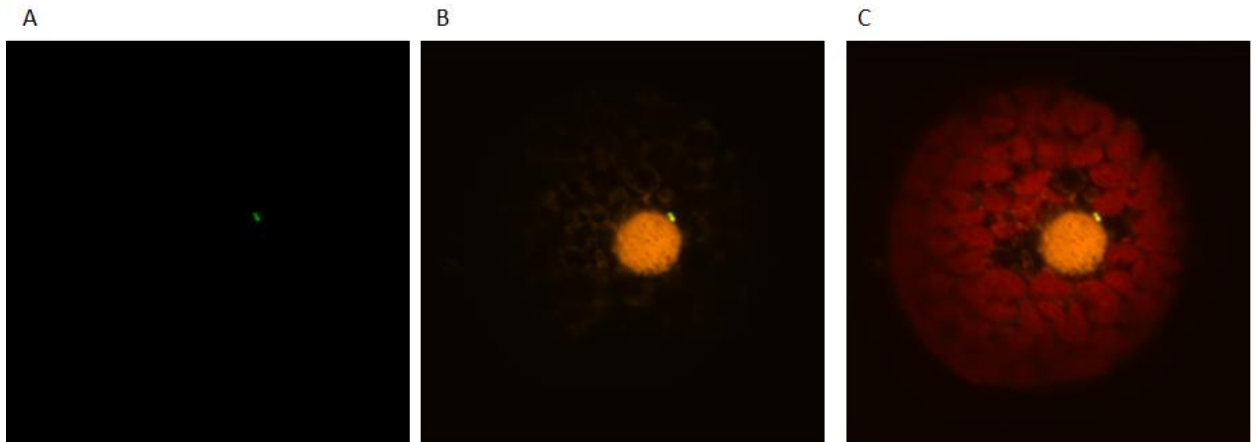
Quantitative real-time PCR (qRT-PCR) analysis being more sensitive than confocal imaging was performed to detect and quantify transcription of the *aadA:gfp* reporter cassette used in the transfection of the microspores and protoplasts mitochondria. The fold difference of normalized *aadA:gfp* mRNA expression, over the housekeeping control elongation factor 1 alpha (*EF1 $\alpha$* ) mRNA expression level, ranged from 0.45 to 0.70 fold in microspores to 32 to 159 fold in protoplasts (Fig. 3.14 and 3.15). This demonstrates that the mitochondria in the transfected microspores and protoplasts were expressing the *aadA:gfp* gene.

The high variation in CT values in the RT-PCR used to analyze each of the three microspore and protoplast transfections performed for each of the five cTPs resulted in high p-values > 0.05 when analysed using the one-way ANOVA method. This means that the transfected microspores and protoplasts were expressing the *aadA:gfp* gene but the



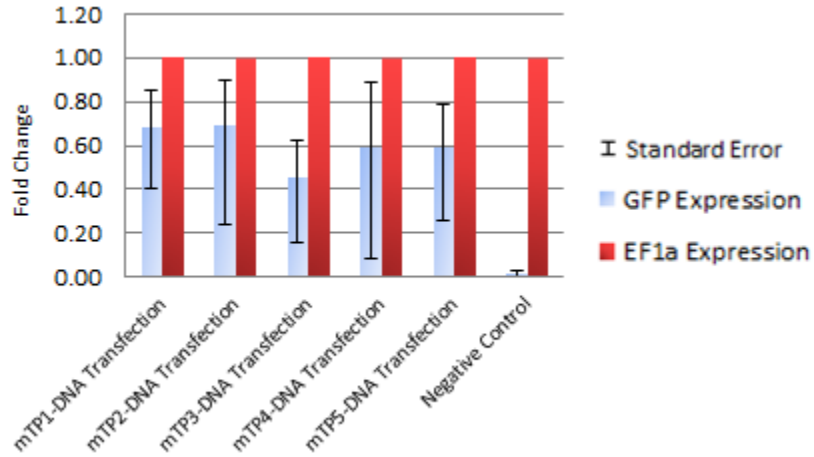
**Fig. 3.12: Confocal image of a protoplast transfected with the *aadA:gfp* reporter cassette using the mTP2 peptide.**

A) Green channel showing the mitochondria expressing the GFP protein. B) Orange channel showing the MitoTracker® Orange CM-H2 TMRos-stained mitochondria. C) A composite image of the red (chloroplast auto- fluorescence), orange (mitochondria) and green (GFP) channels. This image shows the co-localization of the GFP fluorescence with the orange stained mitochondria which appears yellow in the image.



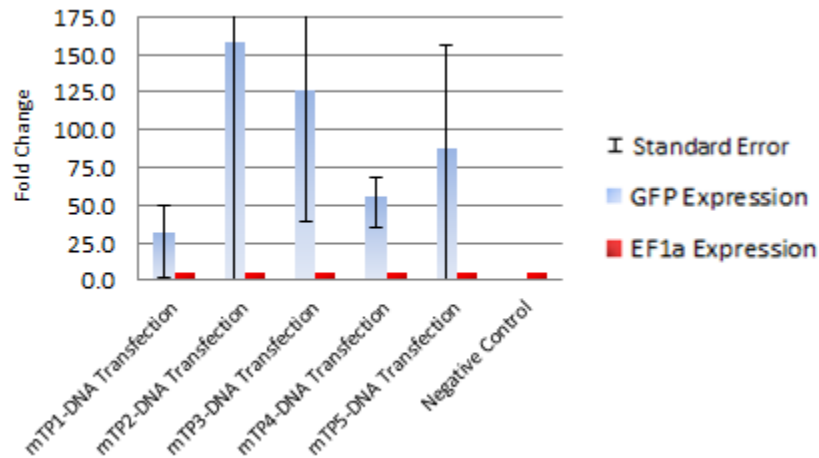
**Fig. 3.13: Confocal image of a protoplast transfected with the *aadA:gfp* reporter cassette using the mTP4 peptide.**

A) Green channel showing the mitochondria expressing the GFP protein. B) Orange channel showing the MitoTracker® Orange CM-H2 TMRos stained mitochondria. C) A composite image of the red (chloroplast auto- fluorescence), orange (mitochondria) and green (GFP) channels. This image shows the co-localization of the GFP fluorescence with the orange stained mitochondria which appears yellow in the image.



**Fig. 3.14: Quantitative real-time PCR (qRT-PCR) analysis of microspores transfected with the five different mTPs 1 -5 delivering *aadA:gfp*.**

Quantitative real-time PCR (qRT-PCR) analysis was used to confirm that the mitochondria of mTP-DNA transfected microspores were expressing GFP. The average of 3 reactions was used to measure the fold difference of normalized *GFP* mRNA expression compared to the control elongation factor 1 alpha mRNA expression levels.



**Fig. 3.15: Quantitative real-time PCR (qRT-PCR) analysis of protoplasts transfected with the five different mTPs 1 -5 delivering *aadA:gfp*.**

The average of 3 reactions was used to measure the fold difference of normalized *GFP* mRNA expression compared to the control elongation factor 1 alpha mRNA expression levels.

mean of the three cTP transfected microspore and protoplast samples CT values measured using RT-PCR weren't significantly different from the negative control.

### **3.3.5 GENERATION OF TRITICALE PLANTS CONTAINING GENETICALLY ENGINEERED MITOCHONDRIA**

Transgenic plants were used to demonstrate that the peptide transfection method could be utilized to stably integrate a transgene into the mitochondrial genome and produce androgenic haploid or double haploid plants with a homoplasmic population of transgenic mitochondria. Isolated microspore culture (IMC) was used for this purpose because this method is compatible with the *in vivo* application of our peptide transfection method and selection protocol and it circumvented the recalcitrance of fertile green plant production from protoplasts. Isolated microspore culture is primarily used to increase the homozygosity of crop plants such as triticale in breeding programs but also increased the probability of producing a fertile homoplasmic plant due to the reduced number of mitochondria within microspores when compared to somatic cells (José and Fernando, 2008; Matsushima et al., 2008).

To test if the mitochondria of green plants could be genetically engineered, mTP1 was used to transfect isolated triticale microspores with the *aadA:gfp* reporter cassette. It was designed with 500 bp of *trnfM-1* gene sequence flanking the left and 500 bp of *rrn18-1* gene sequence flanking the right end of the reporter cassette (Fig. 3.1). These flanking regions of homologous sequence were included in the design of the reporter cassette to support homologous recombination of the transgene in between the *trnfM-1* and *rrn18-1* genes. The *trnfM – rrn18* region is a gene cluster that is repeated 3 times within the *Triticum aestivum* mitochondrial genome (Ogihara et al., 2005). Due to the



high homology shared between the 3 repeat regions it was hypothesised that the reporter gene would recombine into 1 or a combination of the repeat regions of the mitochondria genome within the transfected microspores.

A total of ten green plantlets, *in vitro* selected using streptomycin, were generated from twenty eight separate microspore transfection reactions. The calculated transfection rate per transfection reaction (eight PCR positive plants out of ten total green plants / twenty eight microspore transfection reactions) divided by the number of total green plants (ten total green plants / reaction) was  $2.86 \times 10^{-2}$  transgenic plants / Petri dish.

Two negative microspore culture controls were included to determine the effects that antibiotic selection had on green plant production. These cultures were not treated with peptides nor DNA but were treated with or without streptomycin selection. Only two green plants with streptomycin selection were regenerated from seven microspore culture control reactions containing streptomycin. The calculated regeneration rate of green plants in the presence of streptomycin selection was  $3.17 \times 10^{-2}$  green plants produced with selection / Petri dish. Control plants without streptomycin selection were regenerated but most of them were albino. The small number of green plants that were produced without streptomycin selection died when transferred to soil. Therefore only control plants produced with streptomycin selection were available for comparative analysis.

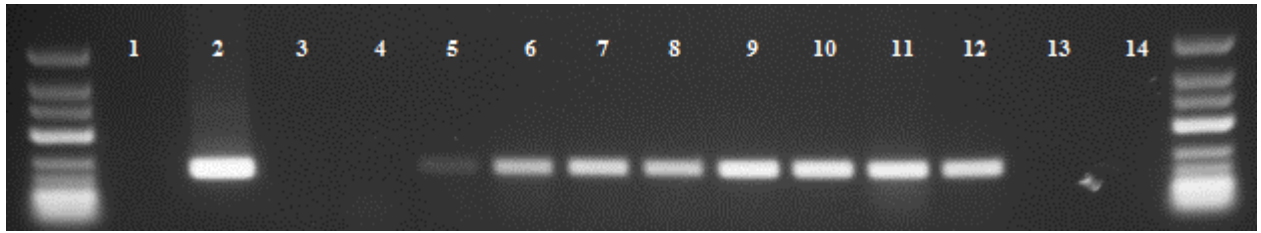
### **3.3.6 MOLECULAR ANALYSIS OF TRITICALE PLANTS CONTAINING GENETICALLY ENGINEERED MITOCHONDRIA**

Genomic DNA was isolated from the leaves of the ten primary transformants ( $T_0$ ) produced from microspores transfected with mTP1 complexed with the *aadA:gfp* reporter cassette and two microspore derived control plants produced with streptomycin selection.

This DNA was screened to evaluate which of the regenerated plants contained the *aadA:gfp* gene. Eight out of the ten plants were characterized as being PCR positive due to the production of an amplicon of the correct size (Fig. 3.16). Plants regenerated from control transfection did not show an amplicon.

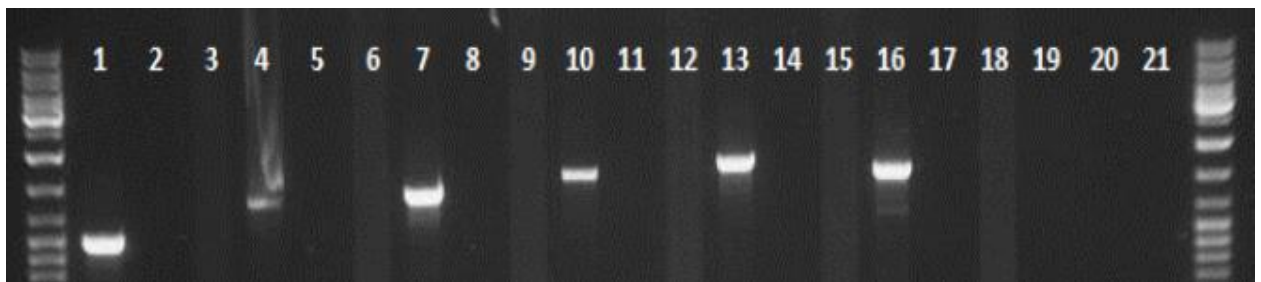
To verify which of the target sites (trnfM-1 – rrn18-1, trnfM-2 – rrn18-2 or trnfM-3 – rrn18-3) the *aadA:gfp* gene integrated into, PCR primers were designed to specifically amplify the left and the right border sequences of each target site if the reporter gene recombined into it (Fig. 3.17). To create the target site specific primers an alignment of the nucleotide sequences from all three of the repeat regions trnfM – rrn18-1, trnfM – rrn18-2 and trnfM – rrn18-3 was done using the bioinformatics software, Geneious version (6.1.3) Biomatters, to generate a consensus sequence. This consensus sequence was used to extend the left and right HR regions of the reporter cassette used in the transfection reactions by 500 bp so that each HR region of the new extended reporter cassette was 1000 bp. The new extended reporter cassette was used to generate target site specific PCR primers. PCR amplification of total genomic DNA produced amplicons of the expected size in eight out of the ten mTP1, *aadA:gfp* reporter cassette transfected microspore regenerated plants. Neither of the two control plants regenerated with streptomycin selection produced amplification products (Fig. 3.17).

Alignments of the amplified left and right border sequence data to the mitochondrial genome and the *aadA:gfp* reporter cassette nucleotide sequences revealed that in two plants (GE-T0-Wm-04 and GE-T0-Wm-10) the cassette had recombined into all three target sites. Three plants had it recombined into two of the target sites trnfM – rrn18 (1 and 3). One plant GE-T0-Wm-08 had it recombined into the trnfM – rrn18-3 target site. Two of the eight plants (GE-T0-Wm-03 and GE-T0-Wm-05) did not produced



**Fig. 3.16: PCR screening for the presence of the *GFP* gene in DNA isolated from green plants tissue regenerated from mTP1, *aadA:gfp* reporter cassette transfected microspores.**

Lane 1 no-template control. Lane 2 Plasmid *aadA:gfp*. Lane 3 GE-T0-Wm-01. Lane 4 GE-T0-Wm-02. Lane 5 GE-T0-Wm-03. Lane 6 GE-T0-Wm-04. Lane 7 GE-T0-Wm-05. Lane 8 GE-T0-Wm-06. Lane 9 GE-T0-Wm-07. Lane 10 GE-T0-Wm-08. Lane 11 GE-T0-Wm-09. Lane 12 GE-T0-Wm-10. Lane 13 Control-01. Lane 14 Control-02.



**Fig. 3.17: T<sub>0</sub> Transgenic Plant #7 GE-T0-Wm-07 PCR screening for targeted gene integration into the *trnM* – *rrn18* (1-3) repeat regions.**

Lanes 1, 4 and 7 correspond to left boarder flanking region amplification products. Lanes 10, 13 and 16 correspond to right boarder amplification products. Lanes 2, 5, 8, 11, 14 and 17 correspond to each primer set being tested against *aadA:gfp* plasmid DNA. Lanes 3, 6, 9, 12, 15 and 18 correspond to each primer set being tested against AC Ultima wild type genomic DNA. Lanes 19, 20 and 21 correspond to no template controls for plant #7, *aadA:gfp* plasmid and AC Ultima wild type genomic DNA.

sequence data with sufficient quality to verify the site of recombination and may explain why PCR amplification products submitted for a number of plants did not yield sequence data (Fig. 3.18 - 3.20 and Table 3.4).

### **3.4 DISCUSSION**

We discovered a new class of CPP and an *in vivo* method to genetically engineer the mitochondria of plant cells while testing the hypothesis - can peptides derived from protein sorting signals and having similar physicochemical properties as CPPs be utilized to transport cargo across the outer plasma membrane into the mitochondria of plant cells? This new class of mitochondrial targeting CPPs were selected from mitochondrial or matrix targeting sequences, which are present at the N-terminus of mitochondrial proteins. They are composed of 10 – 80 amino acids that can form amphipathic alpha helices. There is no or little consensus to the amino acid sequences within these peptides (Bolender, et al. 2008). This has made mTP prediction challenging. There are a number of programs that can identify mTP sequences: TargetP, PSORT II, MITOPRED and MitoProt II (Claros and Vincens, 1996; Nakai and Horton, 1999; Emanuelsson et al., 2000; Guda et al., 2004; Small et al., 2004). TargetP had the highest positive predictive values compared to the other bioinformatics programs at the time of analysis therefore to maximize our chances of predicting true mTP sequences, we used TargetP to analyse all of the downloaded plant protein sequences.

The prediction of mTPs with CPP properties was done using established criteria that impart cell penetrating and mitochondrial targeting properties to peptides and proteins. Interestingly, the physicochemical properties of CPPs, MPPs and mTPs overlap. They all have a high net positive charge due to the large concentration of basic amino



**Fig. 3.18: Image created from DNA sequence alignments of the left and right border sequence data to the mitochondrial genome sequence repeat region 18-1 and the *aadA:gfp* reporter cassette nucleotide sequence.**

A) Image of wheat mitochondrial genomic sequence repeat region18-1 (294591 – 312591) (GenBank accession No. AP008982.1). B) Image of *aadA:gfp* reporter cassette with left and right boarder 500 bp homologous sequence extensions. C) Image of sequence data for T<sub>0</sub> Transgenic Plant GE-T0-Wm-07 amplified left and right border regions. Through sequence comparison of the PCR amplification products to the mitochondrial genome and reporter cassette reference sequences the figure illustrates that the *aadA:gfp* gene perfectly recombined in-between the *trnfM-1* – *rrn18-1* genes within the mitochondrial genome.

**Fig 3.19: Nucleotide alignment of DNA sequence alignments of the left border sequence data to the mitochondrial genome sequence repeat region 18-1 and the *aadA:gfp* reporter cassette nucleotide sequence.**

A) Wheat mitochondrial genomic sequence repeat region 18-1 (294591 – 312591) (GenBank accession No. AP008982.1). B) *aadA:gfp* reporter cassette with left and right border 500 bp homologous sequence extensions. C) Consensus sequence from six different PCR reactions to generate six X sequence data coverage of the T<sub>0</sub> Transgenic Plant GE-T0-Wm-07 amplified left border region. N = no alignment to the other sequences.









**Table 3.4. Sequencing results of the amplified boarder products indicating the sites of targeted gene integration of the *aadA:gfp* gene cassette into the *trnfM – rrn18* (1-3) repeat regions of the mitochondrial genome.**

X= site of *aadA:gfp* gene cassette integration.

<b>Plant Names</b>	<b>trnfM rrn18-1</b>	<b>trnfM rrn18-2</b>	<b>trnfM rrn18-3</b>
<b>GE-T0-Wm-03</b>			
<b>GE-T0-Wm-04</b>	x	x	x
<b>GE-T0-Wm-05</b>			
<b>GE-T0-Wm-06</b>	x		x
<b>GE-T0-Wm-07</b>	x		x
<b>GE-T0-Wm-08</b>			x
<b>GE-T0-Wm-09</b>	x		x
<b>GE-T0-Wm-10</b>	x	x	x

acids such as arginine and lysine within their peptide sequences (Vassarotti et al., 1987; Herce and Garcia, 2008). They commonly form  $\alpha$ -helices and are amphipathic (von Heijne, 1986; Scheller et al., 1999). These concepts and values served well as the basis for our selection criteria but further refinement of what characteristics (length, charge, amino acid composition, secondary structure and hydrophobicity) constitute a CPP or a mTP are needed. These characteristics should then be refined and considered in the overall context of the peptide and how this contributes to the peptide's functional properties.

The organelle colocalization assay served as an effective method to visually screen the thirty one fluorescently labelled peptides. Higher throughput methods like flow cytometry should be considered for future peptide characterization experiments. To be considered as a potential peptide based nucleic acid transduction system required screening of the five mTPs for their ability to bind DNA.

Previous experiments established that the cell penetrating peptide Tat<sub>2</sub> formed a stable complex between the peptide and linearized plasmid DNA using a gel mobility shift assay, nuclease protection assay as well as SEM microscopy to visualize the nanoparticles formed between the peptides and the DNA (Chugh et al., 2009). Our results reiterate Chugh et al., (2009) findings with regard to peptide DNA complex formation (Table 3.3 and Fig. 3.4 – 3.7). Our nuclease protection assay data confirmed that the mTPs have the ability to bind to DNA in a non-covalent manner forming a nanocomplex that protects DNA from nuclease degradation (Table 3.3 and Fig. 3.8 – 3.11). The mTPs capacity to bind DNA and protect it from nuclease degradation were both critical factors in determining if the mTPs could be used as a vehicle to deliver DNA to the mitochondria in isolated plant cells. The gel shift and nuclease protection assays also facilitated the

determination of the minimum peptide concentrations needed to bind to and completely saturate DNA in preparation for the transfection experiments.

In comparison to mTPs, an alternative mitochondrial protein transduction technology that does take cell penetration and DNA binding into consideration is the chimeric protein MTD–TFAM. MTD–TFAM is derived from the CPP, 11-arginine attached to the mTP sequence from malate dehydrogenase. The mitochondria transduction domain (MTD) facilitates the proteins ability to cross the outer plasma membrane as well as target the protein to the mitochondria (Del Gaizo and Payne, 2003; Zhou et al., 2006). The MTD is attached to the protein, mitochondrial transcription factor A (TFAM). TFAM is a mtDNA binding and maintenance protein found in the nucleoid region of mitochondria (Kanki et al., 2004). The recombinant proteins TFAM domain binds nucleic acids and transports them across plasma membranes into the mitochondria of cultured mammalian cells (Keeney et al., 2009). It would be interesting to try the MTD-TFAM technology in cultured plant cells and mTPs in cultured mammalian cells so that a direct comparison of the 2 technologies could be made. The MTD–TFAM protein in principle functions the same as mTPs only it is a much larger protein requiring each separate protein domain to coordinate their functions to achieve mitochondrial transfection.

The five non-labelled mTPs listed in Table 3.3 were tested for their capability to deliver the linearized dsDNA construct encoding the aadA:GFP fusion protein to the mitochondria of triticale mesophyll protoplasts, or microspores using the described reporter cassette transfection assay. Variability in mitochondrial expression determined in the somatic protoplast cells and the germline microspore cells could be explained by the fact that microspores are more difficult to transform and the individual transfection

efficiencies of each peptide varies. Transfection of the mitochondria involves DNA being transported across multiple membranes. The outer plasma membrane is most likely crossed by either direct membrane translocation or one of the endocytic membrane transport mechanism (Madani et al., 2011). This point has yet to be verified experimentally and there is much debate within the scientific community regarding which is the predominant mechanism involved in cell penetrating peptide cargo transduction. Next is the double membrane of the mitochondria. It is unclear precisely how the DNA is entering the mitochondria of the transfected plant cells and will require further investigation in the future to elucidate the uptake mechanism. The DNA could be imported across the mitochondria's double membrane using the mitochondrial protein import pathway. It has also been shown that carrier proteins containing mitochondrial targeting peptide sequences can transport RNAs into the mitochondria (Sieber et al., 2011). Previous research working with isolated mitochondria demonstrated that mitochondria maintain a natural competence to import linear double stranded DNA. The mechanism of import involved the voltage-dependant anion channel (VDAC) and the adenine nucleotide translocator (ANT) (Koulintchenko et al., 2003). It is probable that one of the stated import mechanisms could be involved in the mTP *aadA:gfp* reporter cassette transduction into the mitochondria.

Alternative mitochondrial peptide transfection technologies that have been characterised to transport cargo through the mitochondrial protein import pathway utilized mTP sequences as a functional part in the overall transduction system. Directly conjugating mTP sequences to oligonucleotides (24 bp and 17 – 322 bp) was demonstrated to be an effective mitochondrial nucleic acids transfection method in isolated yeast and rat mitochondria (Vestweber and Schatz, 1989; Seibel et al., 1995).

Mitochondrial import of an mTP conjugated to a peptide nucleic acid (PNA) in isolated mitochondria as well as chemically permeabilized cultured mammalian cells was also demonstrated to transduce cargo into the mitochondrial matrix through the mitochondrial protein import pathway (Flierl et al., 2003). In contrast to the mTP transfection method these approaches are all limited to the transport of small fragments of DNA and the cytotoxicity of membrane permeabilizing chemicals used in the cultured cells.

The protoplast cell culture served as an adequate model system for testing of the mTPs because they can be isolated in abundance with relative ease from leaves and other plant tissues, but they are recalcitrant to whole plant regeneration. A plant tissue culture system that is amenable to fertile green plant production is microspore culture. Knowing that the mTPs could be used to deliver DNA into microspores and have it expressed within the mitochondria, we attempted to engineer the mitochondria of triticale.

Following T<sub>0</sub> plant regeneration from the mTP1 *aadA:gfp* transfected microspores, PCR using *GFP* gene specific primers revealed eight out of the ten plants were characterized as being PCR positive (Fig. 3.16 and 3.17). This indicates that the transfection efficacy for this experiment was low  $2.86 \times 10^{-2}$  transfected plants / Petri dish when compared to green plant regeneration rates of  $4.16 \times 10^{-2}$  green plants / Petri dish from current methods (Asif et al., 2013). In our experience, microspore transformation can be efficient but is quite often variable due to many factors that have yet to be experimentally determined. Within the transfected microspore plant cohort two of the ten generated green plants escaped selection. This may indicate our selective pressure wasn't high enough or a different selectable marker should be used in the future. One might consider the use of the antibiotic resistance gene chloramphenicol acetyltransferase (*cat*). Weimin et al., (2011) inferred that higher plant mitochondria may be sensitive to

chloramphenicol from plastid transformation experiments in tobacco (Li et al., 2011). Chloramphenicol acetyltransferase confers chloramphenicol resistance by acetylating the antibiotic chloramphenicol thereby eliminating its inhibitory effects on protein translation in the mitochondria with no apparent natural resistance in tobacco; making it a potentially effective plant mitochondrial selectable marker (Balbi, 2004). An alternative to antibiotic resistance is the herbicide selectable marker acetolactate synthase (ALS). ALS has been effectively used in monocot nuclear transformation and dicot plastid transformation experiments therefore it may serve as a potential herbicide selectable marker in future plant mitochondrial genetic engineering experiments (Ogawa et al., 2008; Shimizu et al., 2008).

After identifying that eight of the plants were positive for the transgene we wanted to determine where the transgene integrated into the mitochondrial genome. PCR amplification, sequencing, followed by sequence data alignments with the mitochondrial *trnfM* and *rrn18* gene target site nucleotide sequences confirms that the *aadA:gfp* reporter gene recombined into one or a combination of the three *trnfM* – *rrn18* repeat regions (Fig. 3.18 - 3.20 and Table 3.4). Both plastid and mitochondrial DNA recombination and repair proteins share homology as well as putative function between the two organelles therefore gene integration into the mitochondrial genome follows similar DNA recombination and repair pathways as occurs in plastids (Lonsdale et al., 1988; Maréchal and Brisson, 2010). Ultimately the pursuit of achieving transgenic mtDNA homoplasmy in monocots will require identifying an extremely efficient selection protocol that eliminates all wt mtDNA in the first round of plant regeneration or the development of plant tissue culture methods that allows for multiple rounds of selective tissue culture to dilute and eliminate wt

mtDNA during plant regeneration. These methods have proved to be successful in achieving transgenic plastid homoplasmy in dicot crop species (Daniell et al., 2005).

The mitochondria plays a central role in eukaryotic metabolism but due to an inability to genetically modify the mitochondrial genome, many aspects of its involvement in metabolism, cellular physiology, and general cell biology have had to be determined indirectly. The achievement of transient gene expression in triticale mitochondria using short peptides represents an unprecedented opportunity to investigate the transient expression of proteins in plant cell mitochondria *in vivo*. The scientific knowledge gained from the use of this technology could provide vital information related to agricultural and biomedical biotechnology applications in the future.

#### **4.0 SUMMURY**

I tested whether a peptide derived from a plant protein sorting signal sequence possessing similar physicochemical properties as a CPP and could deliver cargo to a targeted subcellular location, e.g. the mitochondria or the chloroplast. Our findings demonstrate that POTCPPs can be utilized to transfect nucleic acids into plant cells in two different triticales cell culture systems. Transient expression was achieved using cTPs in both protoplasts and microspores. Polymerase chain reaction was used to determine if the transgene was present within the cells and qRT-PCR was used to quantify transgene expression. Transgenic haploid plants were generated from mTP1 mediated transfection of triticales isolated microspores, and were screened using PCR. Deoxyribonucleic acid was extracted from the transgenic plants, gene insertion sites were amplified using PCR, and then sequenced. This is the first report of a successful plant mitochondria *in vivo* mediated transfection and an alternative plant cell culture transfection method utilizing peptides. Plant organelle targeting cell penetrating peptides can be used to study transient gene expression in protoplasts or microspores in the monocot species triticales.



## 5.0 FUTURE DIRECTIONS

The two most prominent technologies currently used in nuclear transgenic plant production are *Agrobacterium* mediated gene delivery and particle bombardment. Limitations of these technologies are variable transformation rates, with random nuclear gene integration. These random genetic insertion events often lead to positional effects on transgene expression or epigenetic gene silencing. As a result of the limitations of conventional plant nuclear transformation, investigations into the application of plastid and mitochondrial genetic modification technologies are steadily gaining favour as these methods and technologies mature.

Plastid and mitochondria genetic modification present several advantages over conventional nuclear transformation, with the main advantage being the ability to control the position of transgene integration through homologous recombination, allowing increased control of protein expression. Other advantages include: the ability to express multiple genes as operons can be utilized to introduce or manipulate biosynthetic pathways; higher levels of protein expression can be achieved due to plant cells containing numerous organelles and each organelle possessing multiple genome copies from which the transgene can be transcribed; the absence of gene silencing which is a common problem associated with nuclear transformation; and finally, the majority of plants exhibit maternal organelle transmission thereby acting as a natural biocontainment. Limiting transmission of organelle genetic information through pollen flow reduces the risk of spreading genes from genetically modified (GM) crops to surrounding non-GM species.

The stated benefits are reflected by the success in achieving stable plastid transformation in numerous plant species, the majority of which are dicotyledonous,

using the biolistic gene delivery technology. In spite of this success, stable plastid transformation in monocotyledonous species is still a rare event. This species barrier to monocot organelle transformation shares similarity with respect to the development of *Agrobacterium tumefaciens* mediated gene delivery methods in monocots. Advancements made in plant biology, tissue culture and molecular biology have since overcome these initial obstacles and has led to *Agrobacterium* being adopted as one of the most widely used gene delivery technologies.

To overcome the difficulties associated with plant genetic manipulation, investigators have been exploring the application of peptides to plant transformation. Cell penetrating peptides (CPPs) were demonstrated to have the ability to transduce either independently or covalently linked to cargo into a broad spectrum of cell types including plant cells. Peptide transfection technologies such as CPPs are currently emerging as an alternative viable plant transfection method. The majority of CPP transfection studies have occurred in mammalian cells but over the last ten years plant based CPP experimentation has steadily increased. Initial plant based CPP studies focused on peptide transduction and internalisation mechanisms to determine if CPPs could cross the unique structural feature that differentiates animal cells from plant cells, the cell wall. In these experiments it was demonstrated that several different CPPs could cross the cell wall and the plasma membrane of both dicot and monocot plant species. Further investigations into CPP transduction determined that the peptides could deliver an assortment of macromolecules into plant cells in both a covalent and non-covalent manner. The evolution of plant based peptide transduction technologies are now emerging as nanoscale multi-component macromolecular delivery systems. The nanoparticles or nanocomplexes

are composed of layers of peptides, proteins and cationic lipids that surround the cargo to be delivered.

The POTCPP based gene delivery system targets plant cell organelles using two classes of short peptide carriers. Plant organelle targeting cell penetrating peptides possess three properties 1) cell penetrating 2) organelle targeting and 3) non-covalent cargo binding properties. The peptides form nanoparticles composed of peptides and exogenous double-stranded DNA (dsDNA). The DNA is targeted to the mitochondria of cultured plant cells due to the specific properties of the peptides. These short peptides have cell penetrating properties and they have specific organelle targeting properties which enable the peptides to translocate their cargo across the cell wall and outer plasma membrane of the plant cell and deliver the dsDNA cargo into protoplasts and microspores and have it transiently expressed within the cells.

Based on past peptide transduction experimentation some of the properties involved in CPP transduction have been elucidated. Characterization of the CPP HIV-1 Tat protein led to the identification of the Tat peptide sequence that confers both cell penetrating and nuclear localization properties to the protein. The essential elements responsible for Tat's ability to cross plasma membranes are the guanidinium head group of the arginine residues and the resulting peptide's high net positive charge. Based on the cell penetrating and nuclear localization properties known to be possessed by Tat, it was reasonable to purport that other peptides could have similar cell penetrating and organelle targeting properties.

Further research into the question revealed that protein sorting signals that target proteins into the chloroplast (cTPs) and the mitochondria (mTPs) have similar physicochemical properties as CPPs. They all have a net positive charge due to the large

concentration of basic amino acids such as arginine and lysine within their peptide sequences, they commonly form  $\alpha$ -helices and are amphipathic. These concepts and values served as the basis for our selection criteria to develop POTCPPs.

Further investigation to elucidate POTCPPs internalization mechanisms across the cell wall and plasma membrane, but more importantly across the organelles double membrane, are required to fully characterize the technology. Studying the effects of chemical inhibitors on the different endocytic mechanisms will define which internalization mechanisms predominate. Chemical inhibition will also facilitate the elucidation of how POTCPPs and their cargo enter the organelles. Fluorescence microscopy and the fluorescent labeling of both peptides and nucleic acids will complement the inhibition studies highlighting the mechanisms of peptide transduction. Once the mechanisms are determined, POTCPP transduction could be tested in mutants lacking the functional components necessary for internalization, followed by complementation to restore the functional components.

The development of new peptides or the application of existing peptides to new cell types, requires optimization to achieve high levels of peptide based macromolecular cargo transduction. Variable factors to consider while optimizing the POTCPP technology or performing research on CPPs in general are peptide concentration, amino acid sequence, peptide structure, cargo attachment, cell type and a variable unique to plants, the cell wall.

Titration peptide concentrations will define the minimum amount of peptide needed to protect cargo from nuclease or protease degradation and effectively deliver cargo into cells. The molar ratio of peptide to cargo varies according to which CPP is being tested and what types of macromolecules are being transduced. Gel shift assays and

nuclease or protease protection assays are a quick and easy method to determine what molar ratios of peptide to cargo should be tested in preliminary cellular transduction assays. These tests have been performed on POTCPPs using nucleic acids but not proteins. Determining if POTCPPs could non-covalently transduce proteins into cells would dramatically increase the utility of this delivery system.

Amino acid substitution in the sequence of peptides has been effective at determining what amino acid residues are critical to cellular transduction and what effect these substitutions have on the relationship between structure and activity. Alanine scanning is a common method used in these types of structure activity relationship studies (Elmqvist et al., 2006). Alanine is used to substitute each residue sequentially to identify specific amino acid residues responsible for a peptide's activity. The structural characterization of POTCPPs will provide valuable insight into the functional characteristics of the peptides and how they interact with cargo molecules as well as the mechanisms involved in membrane transduction.

Non-covalent POTCPP cargo attachment was the only cargo delivery method investigated in our study. Covalent attachment to the N or C terminus as well as alternate positions within the POTCPPs sequences may reveal interesting results with regard to transduction efficiencies.

Plant organelle targeting cell penetrating peptides cellular transduction was only investigated in triticales protoplasts and microspores. Microspores' having a reduced number of proplastids and mitochondria to transfect when compared to somatic cell types such as protoplasts increases the probability of achieving homoplasmy in regenerated plants. This is a desirable characteristic; therefore POTCPPs could be tested in other plant species that are amenable to plant propagation from microspores tissue culture. Much

variability exists in how a particular cultivars or different plant species respond to microspores tissue culture. These differences could be exploited to investigate variations in the transfection or selection methods more commonly employed in microspore cultivation in the other species.

POTCPPs could potentially be utilized to genetically engineer the organelles of any cell type with further testing and optimization of the technology. They could have many important applications in cell biology and agricultural biotechnology in both plants and animals. Plant organelle targeting cell penetrating peptide based transfection could be exploited to study the functional roles of organelle encoded genes, to analyze how these genes are regulated, and characterize the function of these gene products in the organelle and in relation to the entire cell. Plant organelle targeting cell penetrating peptides could also be applied in a biotechnology context to produce foreign proteins, altering photosynthetic rates, biomass production, secondary metabolite accumulation, disease resistance, nitrogen assimilation, or the development of therapeutic strategies to treatment human diseases. Peptide based transfection technology has a bright future due its ease of application to cell culture systems and its potential for broad spectrum application in agricultural and biomedical biotechnology.

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