

Western  Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

12-15-2015 12:00 AM

Investigation of Cell-Penetrating Peptide Transformation in Two Regenerable Tissue Culture Systems

Atiyah S. Ferouz

The University of Western Ontario

Supervisor

Dr. Lining Tian

The University of Western Ontario Joint Supervisor

Dr. Danielle Way

The University of Western Ontario

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

© Atiyah S. Ferouz 2015

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Biology Commons](#), [Biotechnology Commons](#), and the [Cell Biology Commons](#)

Recommended Citation

Ferouz, Atiyah S., "Investigation of Cell-Penetrating Peptide Transformation in Two Regenerable Tissue Culture Systems" (2015). *Electronic Thesis and Dissertation Repository*. 3471.

<https://ir.lib.uwo.ca/etd/3471>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

INVESTIGATION OF CELL-PENETRATING PEPTIDE TRANSFORMATION IN
TWO REGENERABLE TISSUE CULTURE SYSTEMS

(Thesis format: Monograph)

by

Atiyah Sumaiyah Ferouz

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Atiyah Sumaiyah Ferouz 2015

Abstract

The genetic engineering of plants allows for the modification of plant genomes, subsequently improving plant traits. There are, however, plants recalcitrant to established transformation methods, requiring the implementation of novel techniques. This study investigates the viability of cell-penetrating peptide (CPP) transformation using Tat₂, a CPP with demonstrated transformation efficiency in plant and animal systems, in two regenerable tissue culture systems, soybean somatic embryos and *Arabidopsis* protoplasts. Assessments of complex formation, Tat₂ cellular translocation, complex uptake, and CPP-mediated transformation were carried out. The results show the formation of a Tat₂-plasmid complex and the uptake of Tat₂ into *Arabidopsis* protoplast cells. However, CPP-cargo complex uptake and successful transformations were not observed in either system. As well, definitive Tat₂ uptake into soybean somatic embryo cells was not detected. This study highlights the areas in each regenerable system where further study is needed for CPP-mediated transformation development.

Keywords

Cell-penetrating peptides, CPP transformation, soybean somatic embryos, *Arabidopsis* protoplasts

Acknowledgments

I would like to thank both of my supervisors, Dr. Lining Tian and Dr. Danielle Way. You have both supported me, in your own ways, and helped me to become a better writer and researcher. I am grateful for all of your encouragement, advice, and direction.

I would also like to thank my advisors, Dr. Rima Menassa, Dr. Alexander Timoshenko, and Dr. Richard Gardiner, for all of your invaluable guidance throughout this process.

To my lab mates in the Tian lab, I don't think I can ever repay all of you for your help with carrying out and troubleshooting my experiments. All of the time and effort you put towards helping me will not be forgotten, I am so lucky to have had the pleasure of working with all of you.

Last, but certainly not least, I would like to thank my extremely loving and supportive family and friends. I don't know how I would have made it through this program without all your love, kindness, and affection.

Table of Contents

Abstract.....	ii
Acknowledgments.....	iii
Table of Contents.....	iv
List of Tables.....	vii
List of Figures.....	viii
List of Appendices.....	x
List of Abbreviations.....	xi
Chapter 1.....	1
1 Introduction.....	1
1.1 Techniques for Gene Transfer.....	2
1.2 Reporter Genes.....	4
1.3 Regenerable Tissue Culture Systems.....	6
1.4 Transformation of Soybean and <i>Arabidopsis</i>	7
1.5 Cell-Penetrating Peptides.....	9
1.5.1 Background.....	9
1.5.2 Cellular entry mechanisms.....	13
1.5.3 Mammalian systems.....	15
1.5.4 Plant systems.....	16
1.6 Research Objective.....	17
Chapter 2.....	18
2 Materials and Methods.....	18
2.1 Preparation of Media and Solutions.....	18
2.1.1 Media for soybean somatic embryogenesis.....	18
2.1.2 Protoplast isolation solutions.....	19

2.1.3	Bacteria culturing medium.....	20
2.2	Soybean Somatic Embryogenesis	20
2.3	<i>Arabidopsis</i> Protoplast Isolation.....	23
2.4	Vector Construction of pDRYFP.....	23
2.5	pBI221 Construct	25
2.6	Tat ₂	29
2.7	Fluorescent Labelling.....	29
2.8	Tat ₂ -pBI221 Complex Formation	31
2.9	Visualizing Uptake of Tat ₂	32
2.9.1	Soybean somatic embryo clusters	32
2.9.2	<i>Arabidopsis</i> protoplasts.....	32
2.10	Visualizing Complex Uptake	33
2.10.1	Soybean somatic embryo clusters	34
2.10.2	<i>Arabidopsis</i> protoplasts.....	34
2.11	CPP-Mediated Transformation	34
2.11.1	Soybean somatic embryo clusters	35
2.11.2	<i>Arabidopsis</i> protoplasts.....	35
2.12	Positive Controls for Transformation.....	36
2.12.1	Particle bombardment in soybean somatic embryo clusters	36
2.12.2	PEG calcium-mediated transformation in <i>Arabidopsis</i> protoplasts.....	37
2.13	Transient Expression Assays.....	38
2.13.1	GUS expression in soybean somatic embryo clusters	38
2.13.2	YFP expression in <i>Arabidopsis</i> protoplasts	39
3	Results	40
3.1	Complex Formation Assessments.....	40

3.1.1	Electrophoretic mobility shift assay.....	40
3.1.2	Confocal imaging of fluorescent complexes.....	42
3.2	Cellular Entry of Tat ₂	44
3.2.1	Soybean somatic embryo clusters.....	44
3.2.2	<i>Arabidopsis</i> protoplasts.....	44
3.3	Uptake of Fluorescently-Labelled Complexes.....	48
3.3.1	Soybean somatic embryo clusters.....	48
3.3.2	<i>Arabidopsis</i> protoplasts.....	50
3.4	Tat ₂ -Mediated Transformation.....	50
3.4.1	Soybean somatic embryo clusters.....	50
3.4.2	<i>Arabidopsis</i> protoplasts.....	53
4	Discussion	60
	References.....	69
	Appendices.....	76
	Curriculum Vitae	78

List of Tables

Table 1: Intracellular FITC-Tat ₂ expression in <i>Arabidopsis</i> protoplasts	47
Table 2: Intracellular FITC and Cy3 fluorescence in <i>Arabidopsis</i> protoplasts.....	52
Table 3: GUS expression in soybean somatic embryo clusters following particle bombardment	55
Table 4: PEG calcium-mediated transformations of <i>Arabidopsis</i> protoplasts.....	59

List of Figures

Figure 1: Cell-penetrating peptide-mediated cargo delivery	12
Figure 2: Stages in the development and isolation of soybean somatic embryos.....	21
Figure 3: Soybean somatic embryo cluster.....	22
Figure 4: Successfully isolated <i>Arabidopsis</i> protoplasts	24
Figure 5: Vector map of synthesized pDRYFP	26
Figure 6: PCR analysis confirming the synthesis of pDRYFP	27
Figure 7: Vector map of pBI221	28
Figure 8: PCR analysis detecting presence of GUS	30
Figure 9: Electrophoretic mobility shift assay of Tat ₂ :pBI221 complexes.....	41
Figure 10: Complex formation between FITC-Tat ₂ and Alexa Fluor 647-pBI221 in solution.....	43
Figure 11: Fluoresceinated Tat ₂ and soybean somatic embryo cells	45
Figure 12: Uptake of fluorescently-labelled Tat ₂ into an intact <i>Arabidopsis</i> protoplast..	46
Figure 13: Fluoresceinated CPP-cargo complexes in the intercellular space between soybean somatic embryo cells	49
Figure 14: Uptake of fluoresceinated CPP-cargo complex components in an <i>Arabidopsis</i> protoplast.....	51
Figure 15: Soybean somatic embryo cluster displaying positive GUS stain after particle bombardment	54
Figure 16: <i>Arabidopsis</i> protoplast after low level CPP-mediated transformation.....	56

Figure 17: *Arabidopsis* protoplast after high level CPP-mediated transformation..... 57

Figure 18: PEG calcium-mediated transformation of *Arabidopsis* protoplast 58

List of Appendices

Supplementary Table 1: FN Lite macro salts and MS micro salts for FN Lite liquid media.....	76
Supplementary Table 2: Summary of CPP transformation experiment parameters in soybean somatic embryo clusters.....	77

List of Abbreviations

*** Standard SI units not listed**

2,4-D: 2,4-Dichlorophenoxyacetic acid

Acp: 6-aminohexanoyl

BIK1: Botrytis Induced Kinase

bp: Base pairs

CaMV: Cauliflower mosaic virus

CPP: Cell-penetrating peptide

Cy3: Cyanine dye 3

DNA: Deoxyribonucleic acid

DR1: Disease Related Gene 1

EDTA: Ethylenediaminetetraacetic acid

EMSA: Electrophoretic mobility shift assay

FN: Finer and Nagasawa

FITC: Fluorescein isothiocyanate

FN: Finer and Nagasawa

GFP: Green fluorescent protein

GUS: β -glucuronidase

HIV: Human immunodeficiency virus

hr: Hour

LB: Lysogeny broth

MES: 2-(*N*-morpholino)ethanesulfonic acid

MS: Murashige and Skoog

nos: Nopaline synthase

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

PTI: Pattern-triggered immunity

psi: pounds per square inch

Tat: Fragment of the trans-activator of transcription for HIV-1

Tat₂: Dimerized form of Tat

Ti: Tumor inducing

UV: Ultraviolet

X-gluc: glucuronide cyclohexamide sodium salt

YFP: yellow fluorescent protein

Chapter 1

1 Introduction

Plants are ecologically and economically valuable organisms that play a significant role in a variety of ecosystem functions and activities. Plants help to prevent soil erosion, serve as material for clothing, and act as a source of food for humans and animals (Groot et al. 2002). These are only a few of the many services that plants provide, but it is clear from this short list that they are indispensable. In a review by Parry et al. (2005) the effects of increased carbon dioxide levels and temperatures on plants, associated with global climate change, were examined. They provided evidence concerning fluctuations in crop production; with cereal production on the decline in certain areas and on the rise in others. They also posited that these disparities will ultimately lead to a shortage in the global food supply, with an emphasis of these effects in poorer regions (Parry et al. 2005). Variations in the production and quality of plants can have a profound effect on the global ecosystem as a whole. The modification of certain plant characteristics may allow for some mitigation of global climate change effects on plant survivability and reproduction.

The human-based alteration of plant characteristics is a practice that has been undertaken for thousands of years. Early civilizations relied upon conventional plant breeding systems based on artificial selection to achieve this goal. The artificial selection of desirable plant traits involves the breeding of individuals within a species to increase and select for the occurrence of a specific characteristic. Increases in crop hardiness, yield, and disease resistance have all been driven by these processes (Halford 2003). Through the years, the

reasons necessitating such alterations have remained the same, while the methods for achieving these goals have changed. Within the last century, techniques were developed that allow us to directly modify aspects of a plant's genome, procedures collectively known as genetic engineering or genetic modification. Genetic modification describes all techniques involving the selective insertion, deletion, or alteration of genes (Slater et al. 2008). These processes have allowed for much more precise modifications to plant traits than conventional breeding systems. They are an invaluable resource in the understanding, development, and manipulation of plant gene functions.

1.1 Techniques for Gene Transfer

There are a number of established methods for the genetic modification of plants. No single method is capable of spanning the large amount of variation presented by the different plants and tissue types to be transformed (De Cleene 1985, Cheng et al. 2004). The methods differ in their mode of gene transfer and possess their own strengths, weaknesses, and characteristics. Although there are multiple distinctions between different methods, they can be divided into two major classes: direct gene transfer and *Agrobacterium*-mediated gene transfer. Direct gene transfer methods are physical or chemical methods for gene delivery, such as particle bombardment and polyethylene glycol-mediated transformation. The *Agrobacterium*-mediated method, as the name suggests, is a method reliant upon the use of bacteria belonging to the genus *Agrobacterium* (Gelvin 2003). This latter technique represents an adaptation of a naturally-occurring phenomenon and is used to an extensive degree in the genetic engineering of plants today.

Agrobacterium tumefaciens is a soil bacterium that causes crown gall disease in several plant species, resulting in the growth of tumor-like structures upon the infected plant (Smith and Townsend 1907). Chilton et al. (1977) reported that a small virulence plasmid was responsible for the induction of tumors after *A. tumefaciens* infection. This plasmid, referred to as the tumor-inducing (Ti) plasmid, became the vector through which genetic engineering endeavors were possible. In 1980, Hernalsteens et al. modified the Ti plasmid and used it to incorporate exogenous DNA into the tumor cells. Harnessing the genetic engineering capabilities of *A. tumefaciens* was a huge milestone and paved the way for future efforts in the field. Under natural conditions, *A. tumefaciens* infects a wide array of dicotyledonous plants (De Cleene and De Ley 1976). Consequently, many monocotyledonous plants were not candidates for this type of transformation (De Cleene 1985). There have been a number of developments in the applicability of the *Agrobacterium*-mediated method on monocots in subsequent years (Hiei et al. 1994). However, the need to genetically engineer cereals and crops such as corn, wheat, and rice led to the exploration of new avenues, such as particle bombardment (Christou 1995).

Particle bombardment, also known as the biolistic method, is the most widely used direct gene transfer method. Klein et al. (1987) demonstrated the use of tungsten microprojectiles and plasmid DNA to induce transient expression of chloramphenicol acetyltransferase activity in onion epidermal cells. Presently, particle bombardment involves the coating of tungsten or gold particles with naked DNA and firing the coated particles at high velocities into the target plant material (Christou et al. 1988). This method is a very effective tool for studying transient expression, whereby exogenous DNA is expressed but does not integrate into the host genome. Stable transformations dependent upon successful

integration of foreign DNA and its subsequent replication are possible but less common with this technique. One of the benefits of particle bombardment is that it lacks the host-specificity of *Agrobacterium*-mediated transformation (Dai et al. 2001). However, the effectiveness of direct gene transfer methods does differ across plants and tissues.

Plant cells have cell walls, which are a formidable barrier to pests, diseases, and genetic modification; some transformation methods require the removal of the cell wall to allow DNA to penetrate into the cells. Protoplasts are produced when plant cells have had their cell walls removed (Cocking 1972). Polyethylene glycol-mediated (PEG) transformation is one of the direct gene transfer methods used for the genetic modification of protoplasts. In 1982, Krens et al. used polyethylene glycol to transform tobacco protoplasts with DNA from the Ti plasmid. Transformation using this method involves treating the protoplasts with a combination of polyethylene glycol and a divalent cation such as calcium. This causes a destabilization of the plasma membrane, rendering it permeable to naked DNA. This method is useful for any species of plant from which protoplasts can be isolated (Lörz et al. 1985, Hayashimoto et al. 1990). The isolation and use of protoplasts is an alternate avenue for plant species recalcitrant to genetic modification in their whole cell form.

1.2 Reporter Genes

Reporter genes are genes whose protein product is easily detectable, either by fluorescence, luminescence, or biochemical staining (de Ruijter et al. 2003). Different reporter genes are used in different situations, each demonstrating effectiveness under different circumstances. Green fluorescent protein (GFP) is a fluorescent reporter gene commonly used in studies of plant transformation. GFP is naturally produced by the luminescent

jellyfish, *Aequorea victoria* (Johnson et al. 1962). Modifications were made to convert this protein for use in plant transformation studies and to increase its potency (Chiu et al. 1996, Davis and Vierstra 1998). Yellow fluorescent protein (YFP) is derived from GFP and is now widely used in plant transformation studies. YFP and GFP fluoresce at different wavelengths, with respective emission peaks of 528 and 504 nm (Wachter et al. 1998). The stable or transient expression of these fluorescent proteins and their respective assays do not damage the plant materials involved. This is demonstrated by their use in studies of localization and visualization of *in vivo* cellular processes (Sheen et al. 1995, Köhler et al. 1997). The use of these particular reporter genes has become very widespread for this reason.

Beta-glucuronidase (GUS) is another reporter gene derived from a natural source. First used as a tool in the study of plant transformation in 1987 this *Escherichia coli* enzyme has become very popular (Jefferson et al. 1987). Modifications of this enzyme were carried out for its use in plant systems (Farrell and Beachy 1990). GUS, unlike GFP and YFP, is not directly visualized. Biochemical assays are carried out whereby the presence of GUS is utilized to convert substrates; the products of this conversion are then detected and measured. Histochemical, fluorometric, and spectrophotometric assays for GUS each require different substrates (Jefferson et al. 1987). GUS and YFP are both genes that can be used as markers for successful transformation and can therefore play a role in the development of existing and novel transformation techniques.

1.3 Regenerable Tissue Culture Systems

Regenerable plant tissues describe all cell and tissue types from which a whole plant can be regenerated. Regenerable tissue culture systems and protocols have been developed around the totipotency of certain cells. Much of the work on plant transformation is carried out on regenerable explants grown in tissue culture, allowing for an *in vitro* growth process that can be controlled and manipulated. The use of certain nutrients and growth regulators allow for specific selection of developmental and regenerable pathways. The explants can be subjected to transformation and then grown into whole plants (Slater et al. 2008). Different systems exist that are known for their regenerative capacities including somatic embryos and protoplasts.

Somatic embryos are embryos derived from somatic tissues. They are very similar to zygotic embryos and go through almost identical morphological and maturational stages (Buchheim et al. 1989). Somatic embryos are created through direct or indirect somatic embryogenesis. During indirect somatic embryogenesis a callus culture is induced from the source explant, the somatic embryos are then formed from this callus culture. Direct somatic embryogenesis does not involve the middle step of callus culture; the embryos are instead derived directly from the source explant. The use of somatic embryogenesis for regeneration is very widespread and the potential for somatic embryogenesis can be found in a variety of tissues and plants. However, the effectiveness of somatic embryogenesis differs not only between plant species but also between genotypes within the species (von Arnold et al. 2002).

The regeneration of whole plants can also come from protoplasts (Roest and Gilissen 1989). Protoplasts, as previously mentioned, are plant cells from which the cell wall has been removed. The generation of protoplasts does not occur under natural circumstances so the cell wall must be removed artificially. Mechanical protoplast isolation methods, involving physical means such as cutting the cells with a knife, were the earliest protoplast isolation methods (Chambers and Höfler 1931). The use of these methods began to dwindle after the discovery of enzymatic protoplast isolation (Cocking 1960). Methods for enzymatic isolation of protoplasts involve the use of enzymes to degrade cell wall components: cellulases to digest cellulose and pectinases to digest pectin. These cell-wall-degrading enzymes are typically mixed with sugars in order to create protoplast isolation solutions (Cocking 1972). There are many plants from which protoplasts can be isolated, increasing the amount of plants that can be genetically modified.

1.4 Transformation of Soybean and *Arabidopsis*

Soybean (*Glycine max*) accounts for approximately 60% of the world's total oilseed production (USDA-FAS 2015). It is an economically important crop and therefore a valuable target for genetic modification. The soybean genome has been fully sequenced, which presents the opportunity for genome alteration (Schmutz et al. 2010). Unfortunately, the recalcitrance of soybean to genetic transformation has hindered progress in this area. Initial attempts at transformation using the *Agrobacterium*-mediated method were met with issues arising from soybean cultivar specificity to *Agrobacterium* infection, leading to low levels of transformation efficiency (Yamada et al. 2012). *Agrobacterium*-mediated transformation of soybean is now most commonly carried out using whole cotyledonary

node explants (Zhang et al. 2014). The biolistic method, an avenue of transformation for troublesome monocots, was also explored for use in soybean transformation. In 1991, Finer and McMullen stably transformed soybean embryogenic suspension culture tissue using particle bombardment. Due to its early success this technique became widely used for soybean transformations.

The effectiveness of many genetic transformation methods is dependent upon the explant used. For soybean, both particle bombardment and *Agrobacterium*-mediated transformation follow this trend. Whole cotyledonary node explants are one of the only explants for which *Agrobacterium*-mediated transformation is successful in soybean. Transformation via particle bombardment, however, can be carried out on multiple soybean explants, with somatic embryos yielding the highest levels of efficiency (Yamada et al. 2012). The development of soybean somatic embryos is cultivar specific due to differences in embryogenic potential, limiting the cultivars that can be used for genetic transformation studies. Jack is one soybean cultivar with proven amenability to soybean somatic embryogenesis (Santarem et al. 1997, Meurer et al. 2001, Tomlin et al. 2002). Jack somatic embryos will serve as one of the model systems in the current study.

Arabidopsis thaliana is a model organism for plants. This small angiosperm has been used in a number of different plant research fields, including studies on plant transformation (Koornneef and Meinke 2010). In 1986, Lloyd et al. transformed *Arabidopsis* leaf disks using *Agrobacterium*-mediated transformation. The use of *Agrobacterium*-mediated transformation has since become a prominent technique for the genetic modification of *Arabidopsis*. The floral dip method, whereby flower buds on *Arabidopsis* plants are submerged in an *Agrobacterium* solution to achieve transformation, is widely used (Clough

and Bent 1998). *Arabidopsis* has a number of benefits for genetic manipulation studies, including small stature, short generation time, a fully sequenced genome, and many established tissue culture systems (Koorneef and Meinke 2010). *Arabidopsis* mesophyll protoplasts, derived from leaf tissue, possess a particularly uniform structure and can be used within minutes of isolation (Yoo et al. 2007). These protoplasts are an established and regenerable *Arabidopsis* tissue culture system, and will be used as the second model system in this study.

1.5 Cell-Penetrating Peptides

1.5.1 Background

Cell-penetrating peptides (CPPs), also referred to as protein transduction domains, are short amino acid sequences that possess the ability to translocate across the cellular membrane (Chugh et al. 2010). Although a peptide sequence must generally be less than 30 amino acids in length to be classified as a CPP, many CPPs are derived from longer peptide sequences or full-length proteins (Lindgren et al. 2000). One of the earliest CPPs was discovered in 1988, when Frankel and Pabo observed the *in vitro* cellular uptake of a full-length human immunodeficiency virus (HIV) protein and its subsequent localization in the nucleus. The protein they observed was the transactivator of transcription (Tat) protein for HIV-1. The identification of the peptides within this sequence that are responsible for Tat's translocation abilities occurred almost a decade later. Various combinations of residues from the original protein, consisting of 86-amino acids, were examined to determine the essential sequence (Vivès et al. 1997). The identification of this short amino acid sequence allowed the development of a Tat CPP. In 1991, Joliot et al. created a polypeptide

mimicking the sequence of the *Drosophila antennapedia* homeodomain. They observed the uptake and nuclear localization of this 60-amino acid sequence in the neuronal cells of rat embryonic tissue. A few years later a peptide consisting of 16 amino acids was created from this original sequence that retained the cellular uptake properties (Derossi et al. 1994); this CPP sequence would later be known as penetratin (Derossi et al. 1998). In both of these examples the CPP was isolated from a naturally occurring source.

Early work on CPPs relied upon the isolation of smaller sequences from larger existing peptides, however, this is just one of the many ways new CPPs can be discovered. Chimeric CPPs are peptides consisting of two or more separate sequences joined together (Chugh et al. 2010). One of the earliest examples of a chimeric peptide is transportan. Transportan is a 27-amino acid sequence synthesized from mastoparan, a wasp venom peptide, and galanin, a neuropeptide (Langel et al. 1996). The uptake of this CPP was examined in Bowes melanoma cells where it was found to be nuclear localized (Pooga et al. 1998). CPPs can also be synthesized entirely, either through various programs used to predict their translocation abilities or simply through trial and error. Polyarginines are synthetic sequences consisting solely of arginine residues that have proven to be effective at translocating across the cellular membrane (Mitchell et al. 2000). Occasionally, these synthetic CPPs display greater effectiveness than their naturally-occurring protein-derived predecessors (Copolovici et al. 2014). In addition, there are artificial CPPs that are not made up of amino acids. Uptake of these artificial CPPs has been observed across multiple cell types. As well, these artificial peptides have displayed lower levels of cytotoxicity than some of the protein-derived CPPs (Farrera-Sinfreu et al. 2005). These examples point to the many possibilities for the discovery, development, and synthesis of CPPs.

CPPs are a very large and diverse group of peptides that come in many shapes, sizes, and charges. As a result, the classification of CPPs can be done a number of ways. Some classification systems are based on structural properties, while others use chemical properties and mechanisms of uptake (Reissmann 2014). Cationic peptides are CPPs that have a net positive charge; they represent the majority of CPPs and include Tat, penetratin, and transportan. The positive charge, in many cases, is conferred by the presence of large numbers of arginine and lysine residues, a trait that Tat possesses (Chugh et al. 2010). Combinations of other cationic amino acids have been tested that do not result in the high levels of uptake demonstrated by Tat or even polyarginines (Mitchell et al. 2000, Wender et al. 2000). Although a net positive charge is common in CPPs, it is not exclusively responsible for their cellular uptake abilities.

The role of CPPs as transporters in biotechnological applications has been a large topic of investigation. It is now known that in addition to being able to move across cellular membranes, CPPs may also translocate attached to macromolecular cargo. CPPs are capable of forming bonds with cargo molecules up to 100 times their molecular weight (Chugh et al. 2010). Cargo may be covalently attached to the CPP or interact via non-covalent electrostatic linkages. The latter form of attachment has demonstrated high transportation efficiency and reduces the amount of interference in cargo bioactivity caused by attachment. Additionally, the process of attaching the cargo non-covalently simply involves incubation of the CPP with the cargo (Fonseca et al. 2009). The formation of a bond, covalent or non-covalent, can occur between the CPP and a wide range of molecules, including drugs, viruses, polymers, and DNA (Jones and Sayers 2012) (Figure 1). The

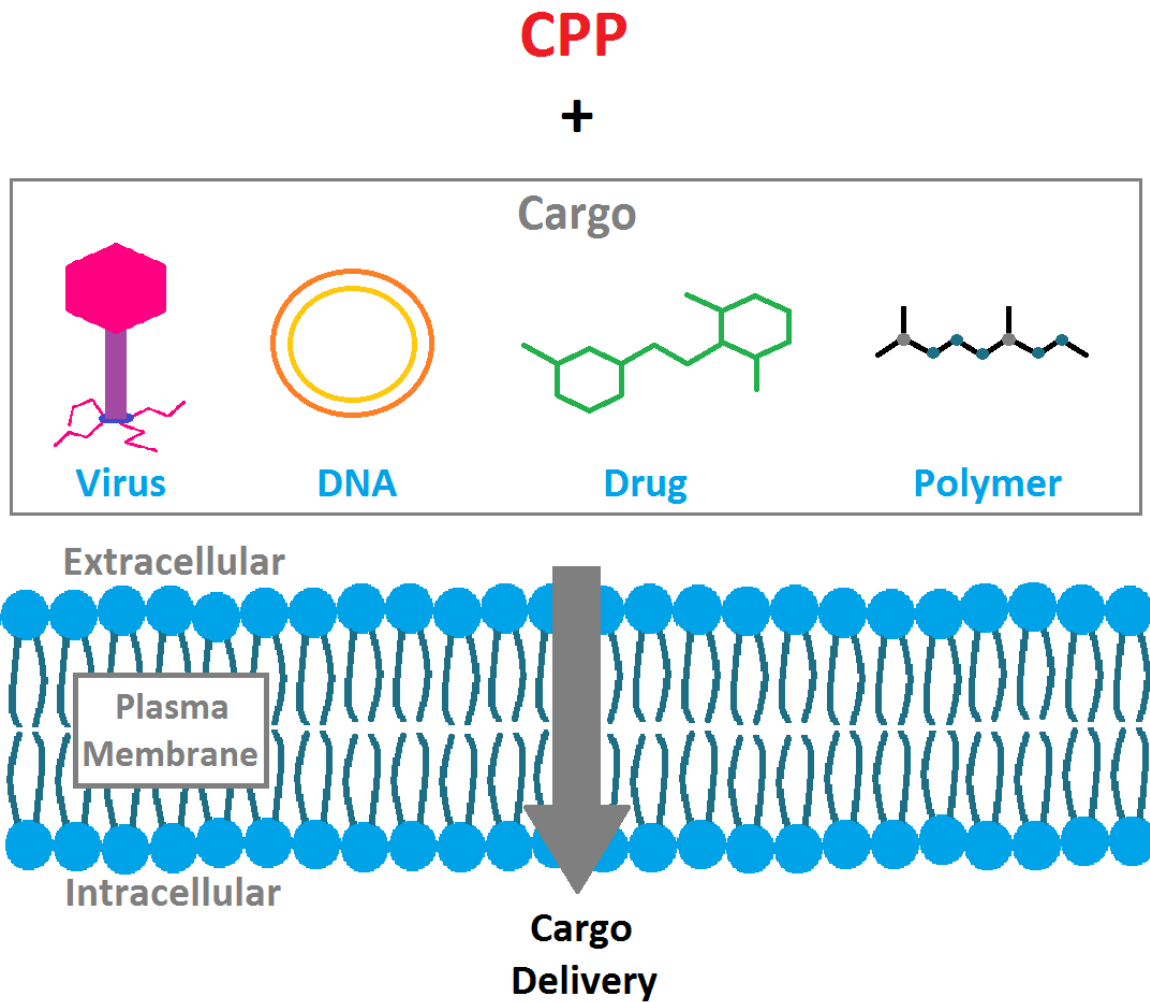


Figure 1: Cell-penetrating peptide-mediated cargo delivery. Cell-penetrating peptides form attachments to various cargo molecules and facilitate their movement across plasma membranes.

DNA transportation abilities of CPPs have led to the study of their potential as tools for genetic transformation.

1.5.2 Cellular entry mechanisms

The mechanisms by which CPPs enter cells have not yet been fully elucidated. There have, however, been a number of proposed mechanisms such as direct transfer and various forms of endocytosis. Direct transfer mechanisms involve the formation of pores in the plasma membrane. Pores formed during direct transfer must subsequently be repaired in order to maintain proper cell functioning. The observed induction of membrane repair responses after cellular entry by CPPs provides evidence for the transient formation of pores and the direct transfer of the CPPs (Palm-Apergi et al. 2009). However, the low levels of cytotoxicity observed during CPP translocation do not support this mechanism (Trabulo et al. 2010). There is evidence of CPP translocation via multiple endocytic pathways, including macropinocytosis and caveolar endocytosis. Caveolar endocytosis, involving small membrane invaginations that typically translocate molecules associated with lipid rafts, was implicated in the cellular entry of CPP-fusion proteins and their subsequent localization within endosomal compartments (Fittipaldi et al. 2003). The cellular uptake of CPPs via macropinocytosis, a form of receptor-independent endocytosis involving extracellular fluid uptake, has also been documented (Wadia et al. 2004, Kaplan et al. 2005). In addition to the evidence of CPP uptake via multiple processes, there are also a whole host of studies providing directly conflicting evidence concerning CPP mechanisms.

CPP uptake experiments have been performed at various temperatures in an attempt to isolate the mechanism of uptake, with mixed results. A temperature of 4 °C is known to

inhibit endocytic cellular processes. In experiments where CPP cellular uptake decreased at 4 °C the conclusion was drawn that endocytic mechanisms were at work (Chugh and Eudes 2008b). However, there are experiments where no decrease in CPP translocation was observed, as well as opposing studies at this temperature where there were increases in CPP translocation (Vivès et al. 1997, Chugh and Eudes 2008a). Results from the use of endocytic and macropinocytotic inhibitors are equally divergent (Chen et al. 2007, Chugh and Eudes 2007, Chugh and Eudes 2008a, Chugh and Eudes 2008b, Chugh et al. 2009, Qi et al. 2011). One of the explanations for this is that the CPP is not responsible for its uptake into the cell. Lundberg et al. (2003) posited that the movement of CPPs into the intracellular space is a result of constitutive endocytosis, a process whereby the cell internalizes proteins on its membrane. The CPP's function in this scenario is merely to adhere to the membrane surface. There is a lot of conflicting research concerning CPP mechanisms, in large part due to observable variations of uptake.

The numerous factors affecting CPP translocation make ascertaining uptake mechanisms difficult. One issue is that attachment to cargo molecules alters the ability of the CPP to enter into cells and the subsequent localization of the complex (Maiolo et al. 2005). This means that a CPP may use certain mechanisms to enter into cells unaccompanied and different mechanisms to enter into cells when attached to cargo. Another issue is that CPPs interact differently across different cell types and tissues. Chugh and Eudes (2008a) studied uptake of multiple CPPs in six different cell types, observing uptake in four out of the six tissues. Levels of CPP uptake differed across these tissues and across the CPPs used. The differences in uptake observed between the CPPs presents another potential problem, as each CPP may use different mechanisms or sets of mechanisms for uptake. It

has also been proposed that CPPs may use different methods to enter the cell depending on the volume of CPP-cargo complexes, with endosomal processes being activated at lower complex concentrations and non-endosomal pathways being activated at higher complex concentrations (Brasseur and Divita 2010). This is just a small subset of the many variables that need to be taken into account when investigating CPP cellular entry mechanisms.

1.5.3 Mammalian systems

Despite the uncertainty regarding CPP mechanisms the use of this technology in mammalian systems is quite advanced. A lot of research on CPPs in these systems has focused on the delivery of drugs and therapeutic agents. The delivery of the chemotherapy drug methotrexate into drug resistant human breast cancer cells has been observed *in vitro* (Lindgren et al. 2006). This was one of many studies that found CPPs could be used to transport drugs into cells that were impervious to other forms of treatment. As well, there have been studies attempting to develop CPP-mediated drug delivery systems that are responsive to specific stimuli such as pH (Sawant et al. 2006). More recently, work on CPPs for drug transport has been carried out *in vivo*. A CPP- and liposome-based delivery system has been successfully used in live nude mice to deliver the anticancer drug doxorubicin and inhibit the growth of tumours (Yang et al. 2014). There have even been a number of human clinical trials for treatments using CPPs as part of a drug delivery system; trials for the use of CPPs in the treatment of psoriasis, myocardial infarctions, and cancer have all been performed (Koren and Torchilin 2012). High levels of success for the use of CPPs in mammalian systems have been achieved, however the same cannot be said for plant systems.

1.5.4 Plant systems

Plant cells and animal cells differ in a number of ways and it is therefore unsurprising that the progress for CPP work in each of these systems is also very different. There is a large discrepancy in CPP uptake when a direct comparison between mammalian cells and plant cells is carried out. The internalization of four CPPs was examined in Bowes human melanoma cells and tobacco protoplasts. CPP uptake for the human cells was greater than 90% for each CPP, while CPP uptakes into tobacco protoplasts were below 16% (Mäe et al. 2005). CPP-mediated plant transformations, using plasmids containing GUS or photoproteins, also display low levels of success, with transient expression levels ranging between 2 and 10% (Chugh and Eudes 2008b, Chugh et al. 2009, Zonin et al. 2011). Furthermore, stable transformation in plants using CPPs is still very uncommon and once again the levels of successful transformation are low (Ziemienowicz et al. 2012). There is potential for the use of CPPs as a tool for genetic transformation in plant systems, however, there remains a large amount of work to do. As mentioned earlier, the uptake of CPPs varies across cell types. The presence of a cell wall further complicates this issue in plant cells. There are approximately 40 different cell types that can be produced by plants, with each possessing different sugars and therefore cell wall compositions (Carpita and Vergara 1998, Ermel et al. 2000, Richmond and Somerville 2001). Cell walls may differ in the types and proportions of polysaccharides they contain (Obel et al. 2009). There are therefore a large number of variables that must be explored in the study of CPP-mediated plant transformation. The development of CPP transformation in plant cells will require investigation of its effectiveness within individual cell types and systems.

1.6 Research Objective

The aim of the current study was to investigate CPP-mediated transformation in two regenerable plant tissue culture systems using Tat₂. The process was broken down and assessed at four major steps; complex formation, CPP uptake, complex uptake, and transformation. In the first step, the formation of a complex between Tat₂ and a plasmid cargo molecule was assessed. The movement of Tat₂ into both soybean somatic embryo cells and *Arabidopsis* protoplasts was then examined. In step three, experiments on the movement of Tat₂-cargo complexes into the same two tissue culture systems were carried out. Finally, CPP-mediated transformations were performed in both tissue systems and transient expression of their plasmid cargos was assayed. The results of this study can act as a guide for future efforts in the development of CPP-mediated transformation of plants.

Chapter 2

2 Materials and Methods

2.1 Preparation of Media and Solutions

2.1.1 Media for soybean somatic embryogenesis

For soybean somatic embryogenesis two media were prepared according to El-Shemy et al. (2004); the Murashige and Skoog (MS) D40 solid medium for somatic embryo induction and the Finer and Nagasawa (FN) Lite liquid medium for somatic embryo proliferation. MSD40 medium contained Murashige and Skoog basal salt mixture (PhytoTechnology Laboratories), 3% (wt/v) sucrose, and Gamborg B5 vitamins (PhytoTechnology Laboratories) in sterile water. Potassium hydroxide was added to achieve a pH of 7.0 before 0.2% (wt/v) Phytigel™ (Sigma) was added. This medium was autoclaved and once cooled 40 mg/L of 2,4-Dichlorophenoxyacetic acid (2,4-D) was added.

FN Lite liquid medium contained FN Lite macro salts and MS micro salts (Supplementary Table 1), 3% sucrose, Gamborg B5 vitamins (PhytoTechnology Laboratories), and 5 mM asparagine in sterile water. The pH was adjusted to 5.8 using potassium hydroxide before autoclaving. After the medium cooled, 5 mg/L of 2,4-D was added.

2.1.2 Protoplast isolation solutions

Preparations for all solutions were carried out according to Yoo et al. (2007). Stock solutions for WI incubation solution, W5 wash solution, and MMG buffer solution were prepared in advance and stored at room temperature. WI incubation solution contained 4 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.7), 0.5 M mannitol, and 20 mM potassium chloride in sterile water. W5 wash solution contained 2 mM MES (pH 5.7), 154 mM sodium chloride, 125 mM calcium chloride, and 5 mM potassium chloride in sterile water. MMG buffer solution contained 4 mM MES (pH 5.7), 0.4 M mannitol, and 15 mM magnesium chloride in sterile water.

The enzyme and PEG-calcium transformation solutions were both prepared fresh shortly before use. The enzyme solution contained 0.4 M mannitol, 20 mM potassium chloride, and 20 mM MES (pH 5.7) in sterile water. This solution was then heated at 60 °C for 5 minutes, after which 1.5% (wt/v) cellulase Onozuka R-10 (Duchefa Biochemie) and 0.4% (wt/v) macerozyme R-10 (PhytoTechnology Laboratories) were mixed in. The solution was then heated for an additional 10 minutes at 60 °C, cooled to room temperature, and 10 mM calcium chloride was added. The PEG-calcium transformation solution was prepared when PEG-mediated transformations were carried out; in each case the solution contained 0.2 M mannitol, 100 mM calcium chloride, and 40% (wt/v) of poly(ethylene glycol) in sterile water.

2.1.3 Bacteria culturing medium

Lysogeny broth (LB) medium was prepared to culture bacteria. Liquid LB medium contained 5 g/L Bacto™ yeast extract, 10 g/L Bacto™ tryptone, and 10 g/L sodium chloride in sterile water. The medium was autoclaved and once cooled 100 mg/L of ampicillin was added.

2.2 Soybean Somatic Embryogenesis

Establishment of soybean somatic embryogenesis followed the method detailed by Finer and McMullen (1991). Soybean plants (cv. Jack) were grown in a growth chamber at 22 °C, 60% relative humidity, and a 16 h photoperiod for 6-8 weeks. Soybean pods (Figure 2A) containing immature seeds of 3-6 mm in size (Figure 2B) were collected. The pods were then surface sterilized with 70% ethanol for two minutes, followed by treatment with a 1.05% hypochlorite solution for ten minutes, and three rinses in sterile water. The seeds were aseptically removed from the pods and the seed coat (Figure 2C) and embryonic axis were excised (Figure 2D). The remaining cotyledons were separated and plated (Figure 2E) on MSD40 embryo induction medium. After 3-4 weeks on MSD40 medium, globular somatic embryos were collected and suspended in FN Lite liquid embryo proliferation medium. These embryos were subcultured every week and maintained in this medium. Embryos proliferated into soybean somatic embryo clusters (Figure 3), approximately 2-5 mm in size consisting of 5-20 individual somatic embryos.

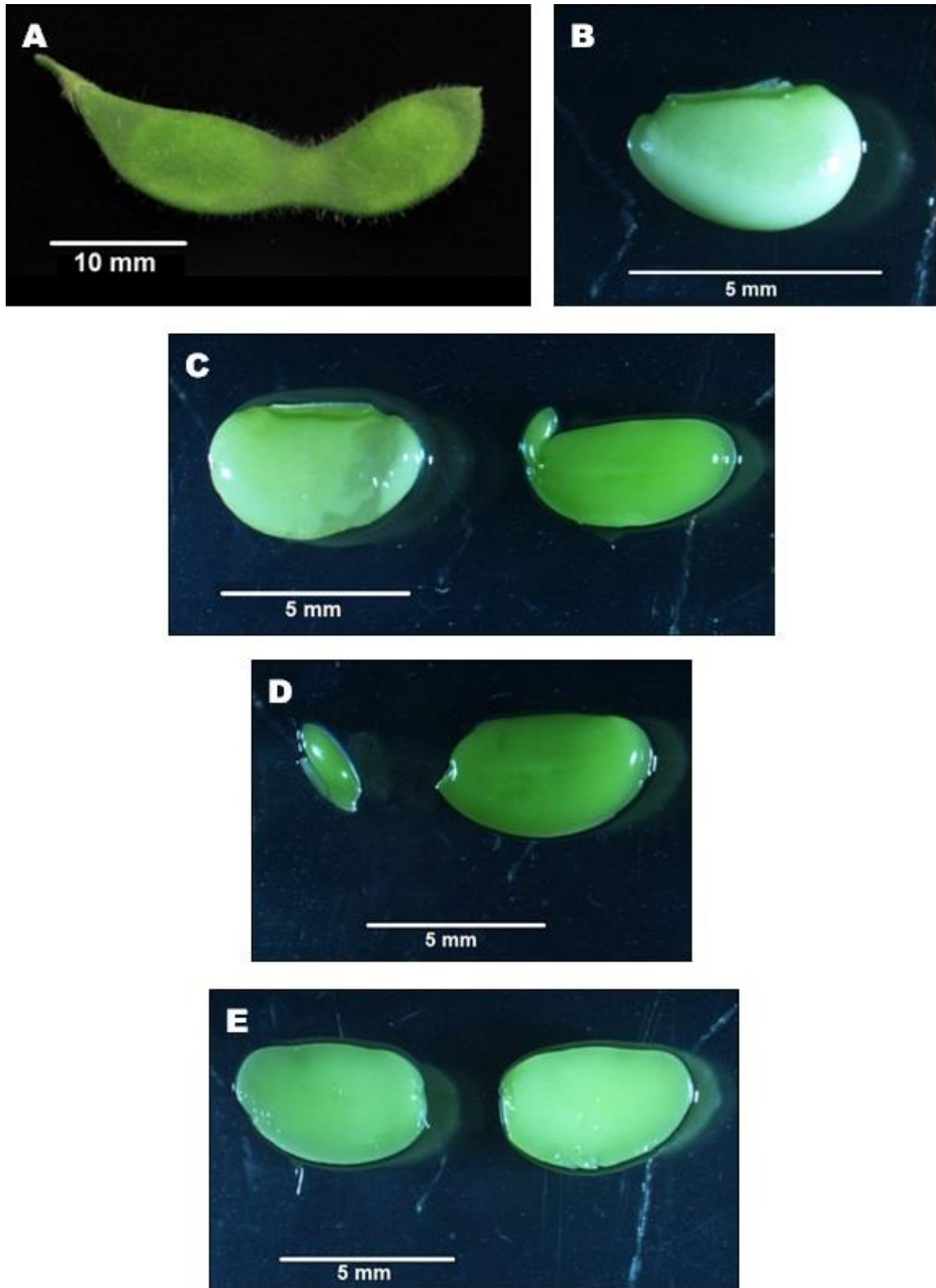


Figure 2: Stages in the development and isolation of soybean somatic embryos. Soybean pods (A) containing immature seeds (B) were collected. The seed coat (C) and embryonic axis (D) were removed, and the cotyledons were plated (E).

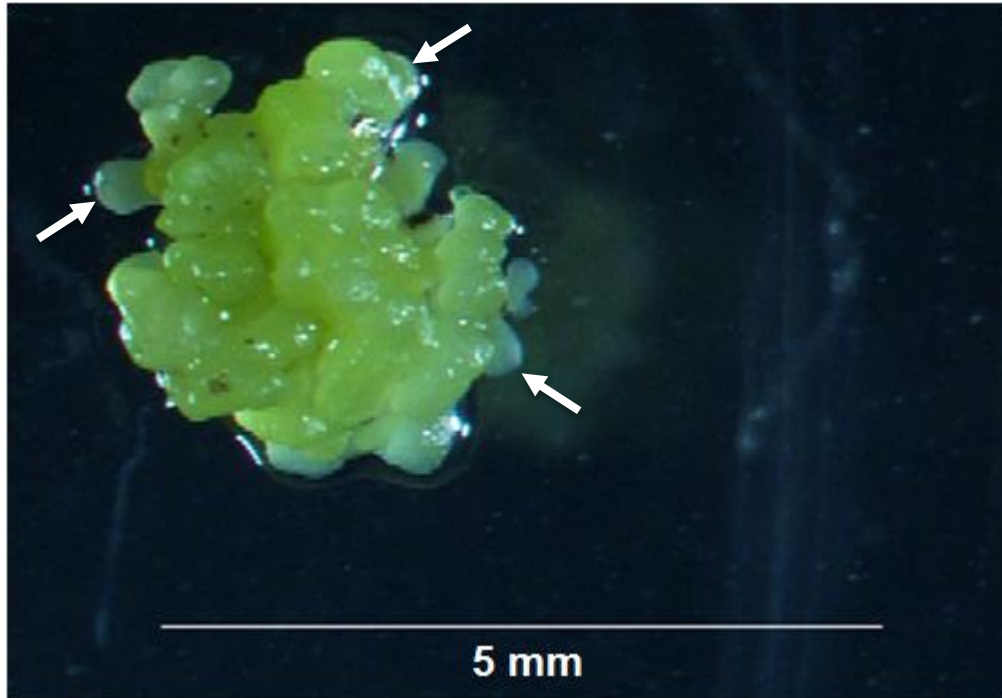


Figure 3: Soybean somatic embryo cluster. Cluster is approximately 3 mm in size and contains about 15 individual somatic embryos, indicated by arrows.

2.3 *Arabidopsis* Protoplast Isolation

Arabidopsis thaliana protoplasts were isolated according to Yoo et al. (2007) (Figure 4). Leaves were removed from 3 to 4 week-old *Arabidopsis* (*Columbia-0*) plants. Leaf strips between 0.5 and 1 mm in size were cut out of the leaves and placed in enzyme solution for cell wall digestion. Digestion was carried out in the dark, with a 30 minute vacuum infiltration followed by a two hour incubation. The enzyme solution containing newly freed protoplasts was then diluted with an equal volume of W5 wash solution. This solution was then filtered through 100- μ m mesh and the flow-through was centrifuged at 100g for 2 minutes in a round-bottomed tube, thus pelleting the protoplasts. The supernatant was removed and the protoplasts were suspended in W5 wash solution. They were then put to rest on ice for 30 minutes, allowing the protoplasts to settle out of the solution naturally. Finally, the supernatant was removed and the pellet was resuspended in 1 mL MMG buffer solution.

2.4 Vector Construction of pDRYFP

For easy visualization of transformation in *Arabidopsis* protoplasts a YFP fusion with Disease Related Gene 1 (DR1), a gene available in Dr. Tian's lab, was constructed. DR1 is a homolog of the *Arabidopsis* gene Botrytis Induced Kinase (BIK1), which localizes in the plasma membrane and plays an important role in Pattern-Triggered Immunity (PTI) signaling (Lu et al. 2010). The fusion was produced using the Gateway® cloning system (Hartley et al. 2000), from pDONR207, a Gateway® entry vector containing DR1, and p2GWY7, a Gateway® destination vector (plasmids courtesy of Dr. Yanjie Luo). The



Figure 4: Successfully isolated *Arabidopsis* protoplasts. *Arabidopsis* leaf cells were subjected to enzyme degradation to create protoplasts. Healthy protoplasts can be seen, with most ranging from 10 to 20 μm in diameter.

resulting plasmid placed DR1 with a YFP C-terminal fusion tag under a Cauliflower Mosaic Virus (CaMV) 35S constitutive promoter and a nopaline synthase (Nos) terminator (Figure 5).

This plasmid, pDRYFP, was then electroporated into DH5 α competent *E. coli* cells using a MicroPulser™ Electroporator (Bio-Rad). Polymerase chain reactions (PCRs) using Go Taq® DNA polymerase (Promega) were performed in an Eppendorf Mastercycler gradient thermocycler (Eppendorf) to confirm the synthesis of pDRYFP. The primers used were provided by Dr. Yanjie Luo, forward primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGGTGCTGCTTAAGTGC-3' and reverse primer 5'-TAATACGACTCACTATAGGGCGA-3'. The PCR reactions were carried out with an annealing temperature of 70 °C, an extension temperature of 72 °C, and an extension time of 2 minutes for 35 cycles.

E. coli cells containing pDRYFP were cultured in liquid LB medium at 37 °C overnight. Extractions of the plasmid from *E. coli* cells were done using the EndoFree® Plasmid Maxi Kit (Qiagen, Hilden, Germany). PCR products were run on a SYBR® Safe stained 1% agarose gel for 1 h, at 100 V in 0.5X TAE buffer (Figure 6).

2.5 pBI221 Construct

pBI221 (Clontech) containing a *uidA* gene coding for GUS, under a CaMV 35S promoter and Nos terminator, was selected for its constitutive GUS expression and small size (5.7 kb) (Figure 7). Plates of *E. coli* cells containing this plasmid were provided by Dr. Sangeeta Dhaubhadel at AAFC London.

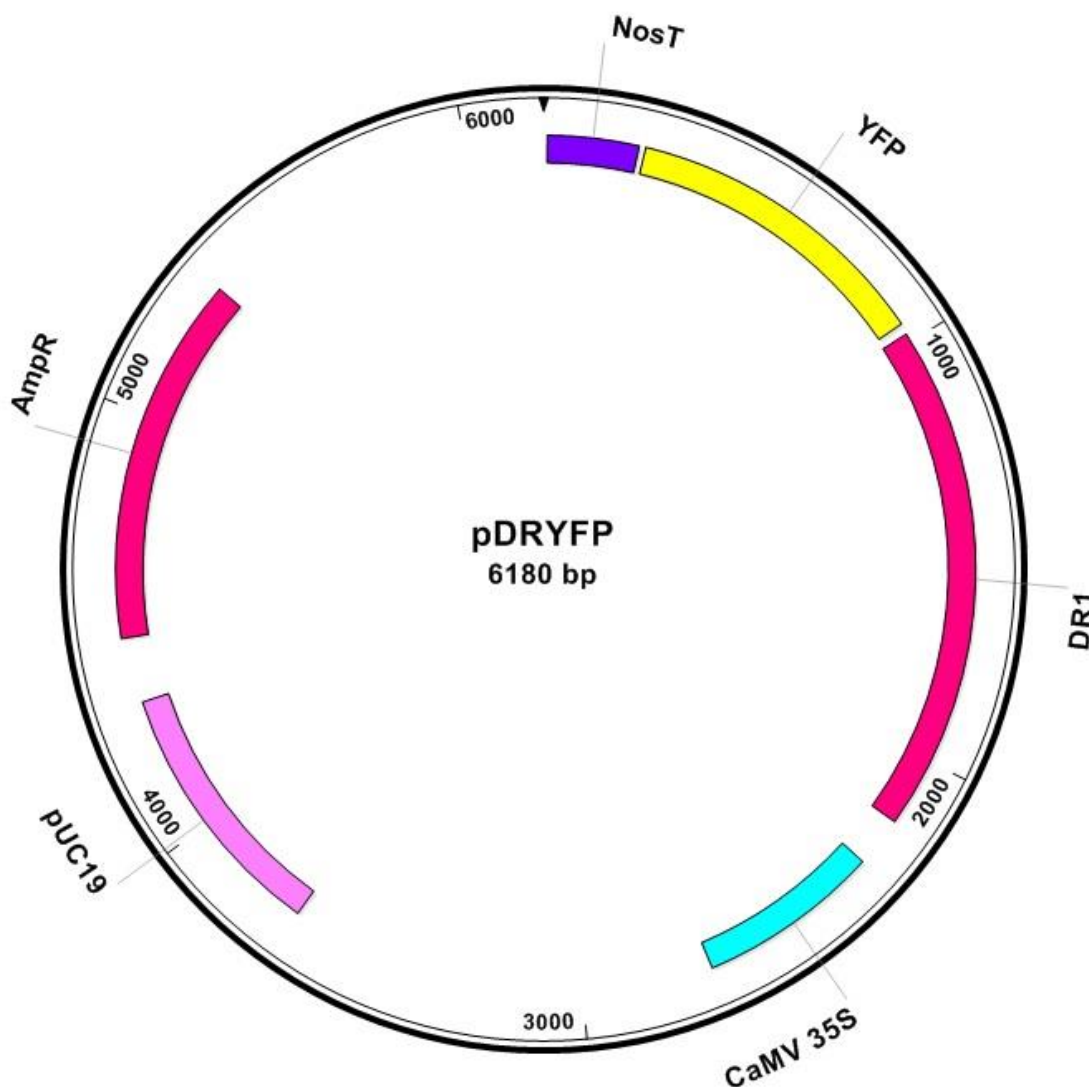


Figure 5: Vector map of synthesized pDRYFP. This vector contains Disease Related Gene 1 with a C-terminal YFP fusion tag under a CaMV 35S promoter and Nos terminator, it was synthesized from p2GWY7 and pDONR207-DR1. The sequence for p2GWY7 was obtained from Plant Systems Biology (<http://www.psb.ugent.be/gateway>). The sequence for Disease Related Gene 1 was obtained from Phytozome (<http://phytozome.jgi.doe.gov>). DNASTAR Lasergene software was used to create the vector map for this plasmid (DNASTAR, Madison, Wisconsin).

YFP, YFP reporter gene; DR1, Disease Related Gene 1; CaMV 35S, cauliflower mosaic virus 35S promoter; pUC19, origin of replication for *E. coli*; AmpR, ampicillin resistance; NosT, nopaline synthase terminator.

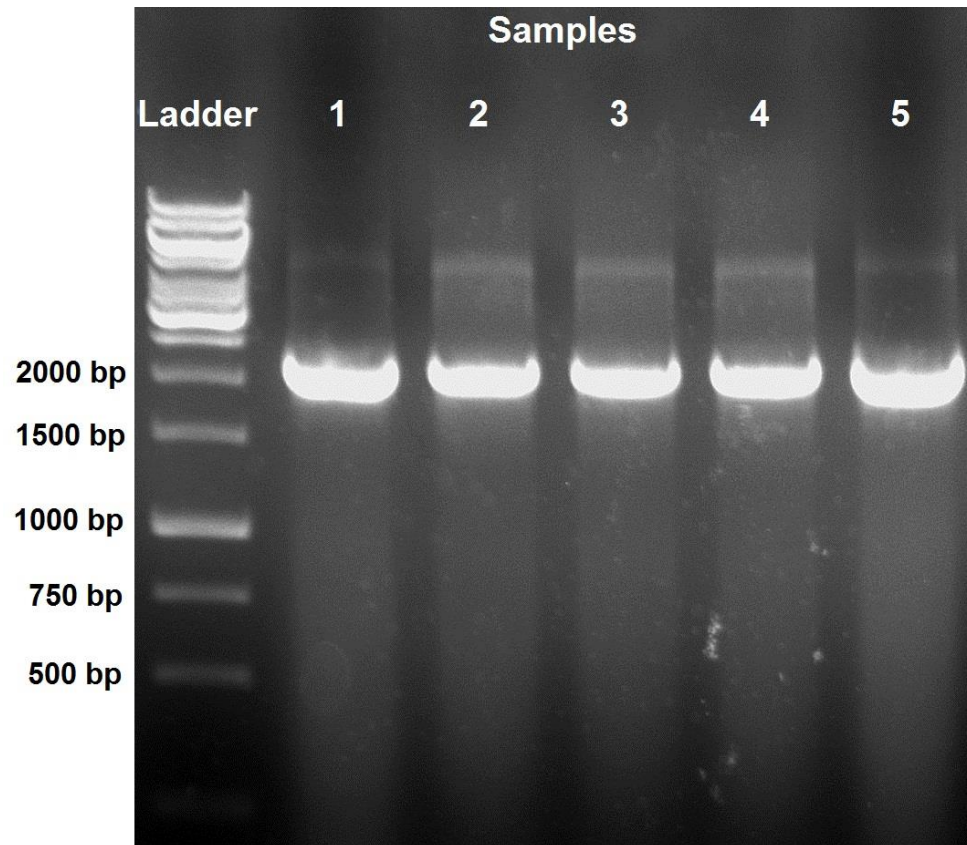


Figure 6: PCR analysis confirming the synthesis of pDRYFP. A PCR was run to confirm the construction of pDRYFP in five samples. The 2168 bp PCR products were run on a SYBR® Safe stained 1% agarose gel.

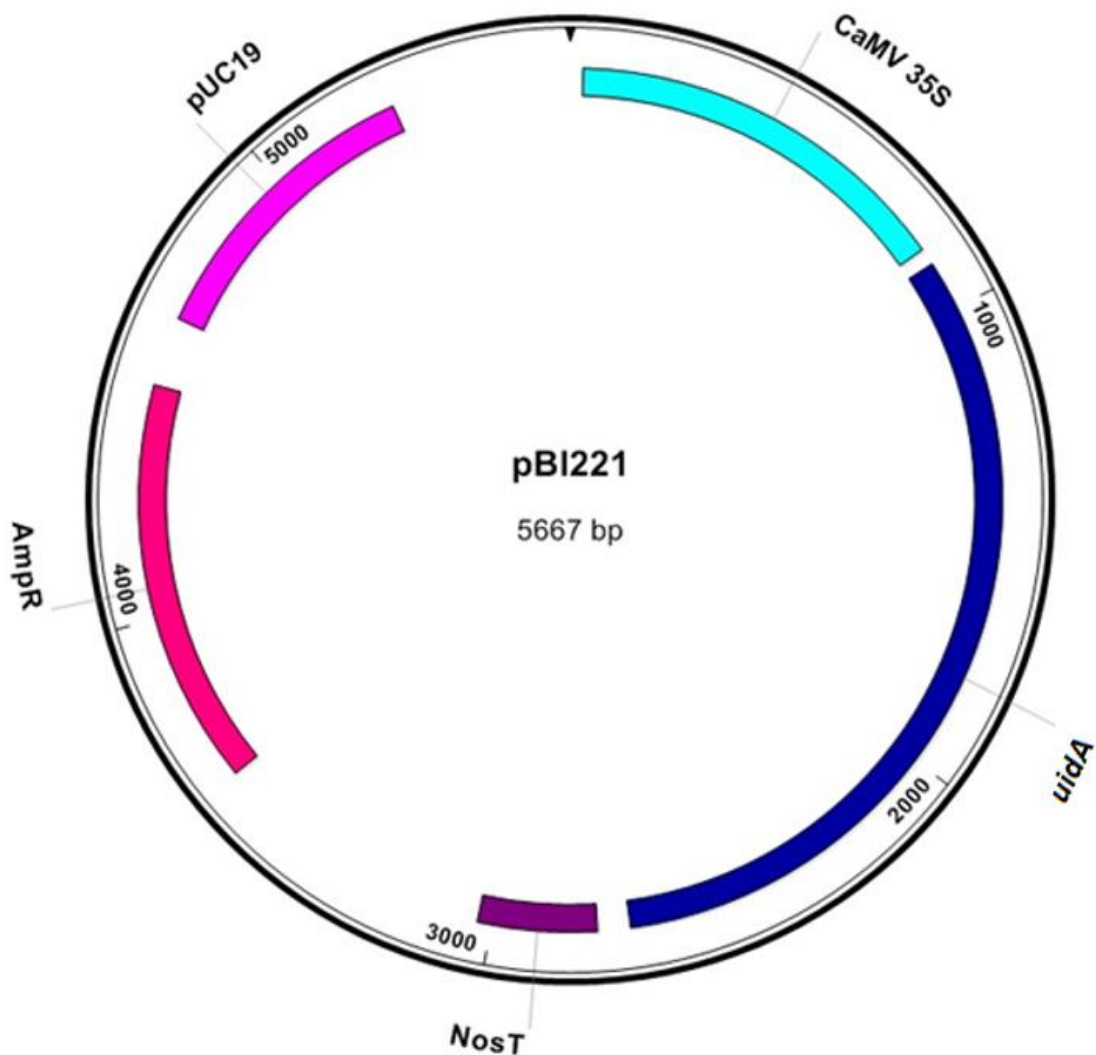


Figure 7: Vector map of pBI221. This vector contains the *uidA* reporter gene coding for GUS expression under a CaMV 35S promoter and Nos terminator. The sequence for this plasmid was obtained from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The vector map was drawn using DNASTAR Lasergene software (DNASTAR).

CaMV 35S, cauliflower mosaic virus 35S promoter; *uidA*, beta-glucuronidase (GUS) gene; NosT, nopaline synthase terminator; AmpR, ampicillin resistance; pUC19, origin of replication for *E. coli*.

Individual bacterial colonies were taken from this plate and cultured in liquid LB medium at 37 °C overnight. A QIAprep Spin Miniprep Kit (Qiagen) was used to extract the plasmid from these bacteria. PCR reactions using Go Taq® DNA polymerase were conducted on ten of the plasmid extractions to confirm that the correct vector had been received, using the forward primer 5'- TACCGTACCTCGCATTACCC-3' and the reverse primer 5'- GAGCGTCGCAGAACATTACA-3' in each. Primers were designed using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and OligoAnalyzer 3.1 (Integrated DNA Technologies). The PCR reactions were carried out in an Eppendorf Mastercycler gradient thermocycler with an annealing temperature of 60.75 °C, an extension temperature of 72 °C, and an extension time of 30 seconds for 35 cycles. PCR products were run on a 1% agarose gel stained with SYBR® Safe for 1 h, at 100 V in 0.5X TAE buffer (Figure 8).

2.6 Tat₂

Initial stocks of Tat₂ were provided by Dr. Francois Eudes at (Agriculture and Agri-Food Canada) AAFC in Lethbridge, AB. Additional stocks were ordered using the same sequence (RKKRRQRRRRKKRRQRRR). All stocks of Tat₂ were synthesized by CanPeptide (Montreal, Quebec, Canada).

2.7 Fluorescent Labelling

pBI221 was labelled with Alexa Fluor 647 using a ULYSIS Nucleic Acid Labeling Kit (Thermofisher Scientific). pDRYFP was labelled with a cyanine dye (Cy3) using a Mirus Label IT® Tracker Intracellular Nucleic Acid Localization Kit (Mirus). Tat₂ with an N-

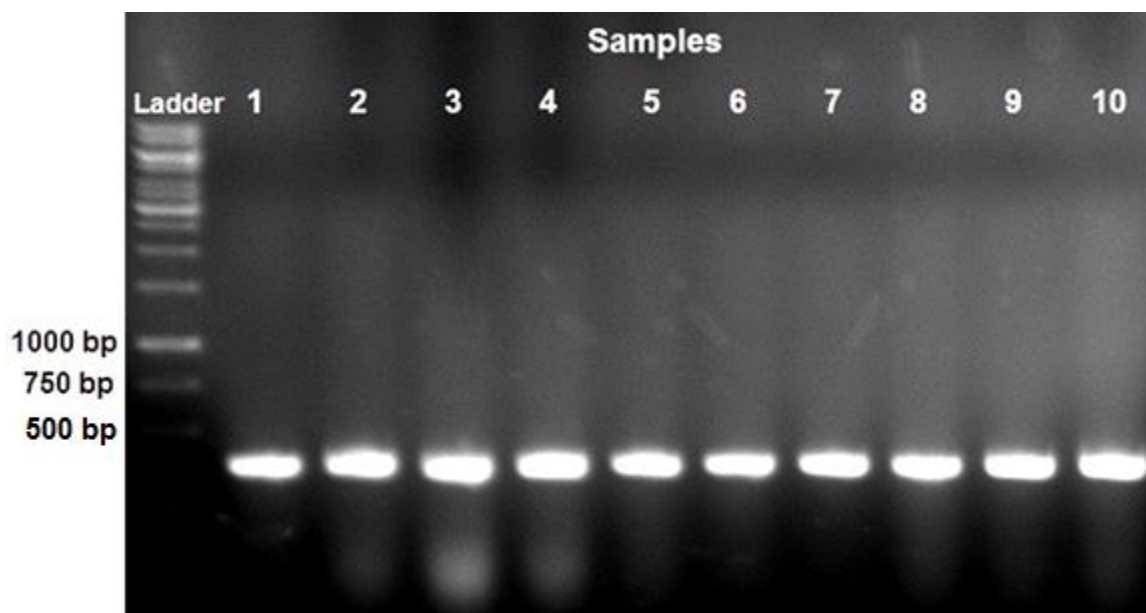


Figure 8: PCR analysis detecting presence of GUS. The presence of GUS was assessed by PCR in ten plasmid extractions to confirm that the plasmids were pBI221. The 376 bp PCR products were then run on a SYBR® Safe stained 1% agarose gel.

terminal fluorescein (FITC) label and a 6-aminohexanoyl (Acp) linear spacer (FITC-Acp-RKKRRQRRRRKKRRQRRR) was synthesized by CanPeptide.

2.8 Tat₂-pBI221 Complex Formation

In order to investigate the formation of a complex between Tat₂ and pBI221, an electrophoretic mobility shift assay (EMSA) was carried out according to Chugh and Eudes (2008b). Eight different CPP to plasmid ratios were investigated: 0.5:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, and 7:1 (w/w). For every combination 1 µg of pBI221 was used and the corresponding amount of Tat₂. Components for each combination were individually prepared in 25 µL of sterile water before being combined and creating a final volume of 50 µL. Once combined, the components were incubated for 1 h at room temperature to allow for complex formation. Loading dye was added in a 1:5 ratio (v/v) with the complex solutions before 12 µL of each solution was run on a gel. Electrophoresis was carried out using a 0.8% agarose gel stained with SYBR® Safe. This gel was run at 100V for 1.5 h in 1X TAE buffer. The control consisted of 1 µg of pBI221 in 50 µL of sterile water.

A single CPP to plasmid ratio was investigated for fluoresceinated complex formation. CPP and plasmid components were combined at a ratio of 4:1, using 4 µg of Tat₂ and 1 µg of pBI221. This ratio was selected due to its effectiveness in other work on CPP-mediated plant transformation (Chugh and Eudes 2008b, Ziemienowicz et al. 2012). Fluorescently-labelled Tat₂ and fluorescently-labelled pBI221 were prepared individually in 10 µL of sterile water. These components were then combined for 1 h at room temperature and imaged using a Leica TCS SP2 confocal laser scanning microscope (Leica Inc.). Tat₂ had

a FITC label (green, 494 nm excitation peak) and pBI221 had an Alexa Fluor 647 label (blue, 650 nm excitation peak).

2.9 Visualizing Uptake of Tat₂

Plant materials were incubated with fluorescently-labelled Tat₂ for 1 h, in order to observe cellular entry of fluoresceinated Tat₂. Plant material was imaged using a confocal laser scanning microscope and merged images show localization of Tat₂ in relation to plant structures. Negative controls with sterile water were also carried out.

2.9.1 Soybean somatic embryo clusters

Soybean somatic embryo clusters were treated with 200 µL of sterile water containing 4 µg of FITC-Tat₂. Embryo clusters were then rinsed with sterile water three times and immersed in a 4% agarose solution. Once the agarose solidified, the clusters were sliced into 35 µm slices using a Leica VT 100S vibratome (Leica Inc.). Three replicates were conducted in total, consisting of five embryo clusters each. Eight slices were taken from each cluster and imaged, for both treatment and controls.

2.9.2 *Arabidopsis* protoplasts

In each replicate, 300 µL of MMG solution containing *Arabidopsis* protoplasts were treated with 20 µL of sterile water containing 8 µg of FITC-Tat₂. Protoplasts were washed by adding 900 µL of W5 wash solution and rocking the tubes back and forth. The solution was then centrifuged at 100g for 2 minutes, the supernatant was removed, and the protoplasts were suspended with 250 µL WI incubation solution in the well of a 24-well

tissue culture plate. A filter for chlorophyll autofluorescence (red, 685 nm excitation peak) was used during imaging and protoplasts displaying internalized Tat₂ were evaluated. Three replicates were performed, and 100 protoplasts were examined in each replicate, including controls.

2.10 Visualizing Complex Uptake

The fluoresceinated CPP and respective plasmids, pBI221 for soybean somatic embryo clusters and pDRYFP for *Arabidopsis* protoplasts, were combined to form complexes. Different plasmids were used to maximize the visualization of the complex within the respective plant materials. Observations of YFP fluorescence within each plasma membrane of soybean somatic embryo cells would not be feasible given the layout and overlap of the cells. In addition, treatment of *Arabidopsis* protoplasts with the substrates involved in GUS detection could lead to changes in the osmotic potential resulting in damage to the protoplasts. The amount of Tat₂ and plasmid used did remain constant however; in each case 8 µg of the CPP and 2 µg of the plasmids were combined, maintaining a 4:1 CPP:plasmid ratio. The components were prepared separately in sterile water and then placed together for 1 h. After the complexes had formed the plant materials were incubated in the solutions for 1 h to allow for complex uptake into the cells. Images were taken using a confocal laser scanning microscope, and merged images were used to observe complex localization in relation to plant cell structures. Negative controls consisting of treatment with sterile water or plasmid alone were also performed.

2.10.1 Soybean somatic embryo clusters

FITC-labelled Tat₂ and Alexa Fluor 647-labelled pBI221 were each prepared in 100 µL of sterile water, after which they were combined. Soybean somatic embryo clusters were treated with the 200 µL solution and then encased in 4% agarose. These clusters were then sliced and imaged as previously described (refer 2.9.1). Three replicates were carried out in total, consisting of eight slices of five embryo clusters for each treatment and control.

2.10.2 *Arabidopsis* protoplasts

FITC-labelled Tat₂ and Cy3-labelled pDRYFP (magenta, 550 nm excitation peak) were prepared separately in 10 µL of sterile water. The components were combined and used to treat 300 µL of MMG solution containing *Arabidopsis* protoplasts. Protoplasts were then rinsed, centrifuged, suspended in WI incubation solution, and imaged as previously described (refer 2.9.2). Three replicates were performed with 100 protoplasts evaluated in each treatment and control.

2.11 CPP-Mediated Transformation

CPP-mediated transformation was carried out according to Chugh and Eudes (2008b). The Tat₂ and plasmid components were prepared separately in sterile water; unlabeled components were used. Components were combined for 1 h at room temperature to allow for complex formation. Plant materials were then incubated with the complexes to allow for transformation of the cells. Negative controls consisting of treatment with sterile water or plasmid alone were also carried out.

2.11.1 Soybean somatic embryo clusters

Embryo clusters underwent CPP-mediated transformations using various parameters (Supplementary Table 2) to determine which factors had an effect on the transformation rate. In all experiments Tat₂ and pBI221 were used, however, there were variations in the ratio of CPP to plasmid and the amount of CPP and plasmid. Tat₂ to pBI221 ratios of 3:1, 4:1, 5:1 and 6:1 were investigated, with a ratio of 4:1 maintained in the majority of the experiments. Different plasmid amounts of 1, 3, 4, and 5 µg were examined, altering the concentration of complexes in treatment solutions. Regardless of ratio and amount, each component was individually prepared in 100 µL of sterile water.

Once components were combined and complex formation took place, the embryo clusters were treated with the complex solution. The amount of time the clusters were exposed to treatment is the incubation time. Incubation times of 1 h were used for the majority of the experiments; however, 2, 3, and 4 h were also examined. Additionally, temperatures of 24 °C, 33 °C, and 37 °C during incubation were assessed to see if there was any effect. After embryo clusters were treated with complex solutions they were rinsed three times with sterile water and re-suspended in FN Lite liquid embryo proliferation medium. Twenty somatic embryo clusters were evaluated per treatment and controls.

2.11.2 *Arabidopsis* protoplasts

Two sets of CPP-mediated transformations were conducted in *Arabidopsis* protoplasts, one with a low level of Tat₂ and pDRYFP (2 µg and 8 µg, respectively), and one with a high level of Tat₂ and pDRYFP (20 µg and 80 µg, respectively). For the low level

transformations, each component was prepared separately in 10 μL of sterile water and then combined, forming 20 μL of solution. For the high level transformations, both components were combined in 100 μL of sterile water, maintaining a minimum amount of liquid. In each case, after the complexes were given time to form, the solutions were applied to 300 μL of MMG solution containing *Arabidopsis* protoplasts for 1 h. W5 wash solution was then added and the tubes were rocked back and forth to end the transformation process. Protoplasts were centrifuged at 100g for 2 minutes, the supernatant was removed, and the protoplasts were re-suspended in 250 μL of WI incubation solution. One hundred protoplasts were evaluated per treatment and control; three replicates were carried out for each type of transformation.

2.12 Positive Controls for Transformation

2.12.1 Particle bombardment in soybean somatic embryo clusters

Materials and protocol for particle bombardment were provided by Dr. Rima Menassa at AAFC London. Gold particles were used as the microcarriers in these experiments, 50 μL of gold particles suspended in 50% glycerol (50 mg/mL) were transferred to a siliconized microcentrifuge tube and vortexed. During vortexing 5 μg of pBI221, 50 μL of 2.5 M calcium chloride, and 20 μL of 0.1 M spermidine were sequentially added. The solution was vortexed for another 20 minutes and then centrifuged at 7000g for 1 minute. The supernatant was discarded and the pellet underwent two ethanol wash steps, in both steps the pellet was rinsed with ethanol and centrifuged at 7000g for 1 minute, after which the supernatant was removed. In the first step 70% ethanol was used and in the second step

100% ethanol was used. After which, there was a final resuspension of the DNA-coated gold particles in 50 μ L of 100% ethanol.

Bombardments were conducted using the Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad) in a sterile environment under conditions described by Khalafalla et al. (2005). The macrocarriers, macrocarrier holder, and stopping screens were sterilized in 70% ethanol and dried. For each replicate, the macrocarrier was placed in the center of the macrocarrier holder and 10 μ L of pBI221-coated gold particles were applied, which was then left to dry. The helium gas tank was brought up to a 1100 psi value. The rupture disk was rinsed in isopropanol and placed in the rupture disk retaining cap; this cap was then attached to the gas acceleration tube. The stopping screen and the macrocarrier holder, containing the coated macrocarrier, were placed in the microcarrier assembly with the macrocarrier facing the stopping screen. The petri dish containing soybean somatic embryo clusters was then placed into the base of the chamber 6 cm from the macrocarrier. Once the chamber was sealed, a vacuum was created inside and firing occurred. The chamber was then vented and the petri dish was removed. Soybean somatic embryo clusters were returned to FN Lite liquid embryo proliferation medium. Three replicates were performed for this positive control of pBI221 transformation.

2.12.2 PEG calcium-mediated transformation in *Arabidopsis* protoplasts

Polyethylene glycol (PEG) calcium-mediated transformation was conducted according to Yoo et al. (2007). In each treatment, 300 μ L of MMG solution containing *Arabidopsis* protoplasts and 20 μ g of pDRYFP in 20 μ L of sterile water were combined. This mixture

was then treated with 110 μL of PEG-calcium transformation solution for five minutes. To end the transformation process 900 μL of W5 wash solution was added and the tubes were rocked. This mixture was then centrifuged at 100g for 2 minutes and the supernatant was removed. Protoplasts were then re-suspended in 250 μL of WI incubation solution. Six replicates were conducted for this control, three of which consisted of counting 100 protoplasts each and three of which consisted of counting 300 protoplasts each.

2.13 Transient Expression Assays

Assays were carried out after the transformations to determine if there was any transient expression of the reporter genes contained within each plasmid, indicating successful transformation. For both CPP-mediated transformations and positive controls, the respective transient expression assays remained the same within each tissue culture system.

2.13.1 GUS expression in soybean somatic embryo clusters

The transient expression of GUS was assayed in soybean somatic embryo clusters transformed with pBI221, three days after CPP-mediated transformation or particle bombardment. The GUS histochemical assay was carried out using the methods of Jefferson et al. (1987). Soybean somatic embryo clusters were incubated in a glucuronide cyclohexamide sodium salt (X-gluc) solution containing 1 M sodium phosphate (pH 7.0), 0.5 M ethylenediaminetetraacetic acid (EDTA), 10% Triton X-100, 50 mM potassium ferricyanide, 0.1 M X-gluc, and 20% (v/v) methanol in sterile water. Embryo clusters were vacuum infiltrated for 30 minutes before being incubated overnight at 37 °C. Treatment with a 95% ethanol solution was then carried out to remove the clusters' natural

pigmentation and highlight any staining. This stain allows for a qualitative assessment of the presence of GUS in the clusters. Positive staining is indicated by the appearance of blue areas on the tissue.

2.13.2 YFP expression in *Arabidopsis* protoplasts

Assays of YFP expression were carried out on protoplasts after a 17 h incubation in incubation solution. This was done by imaging the protoplasts with a confocal laser scanning microscope using a YFP filter (yellow, 514 nm excitation peak). A filter for chlorophyll autofluorescence was also used and images were merged to observe localization in relation to plant organelles.

3 Results

3.1 Complex Formation Assessments

Complex formation studies were carried out to ensure binding between the CPP and cargo, the first step in CPP transformation. Two assessments for complex formation were performed, an electrophoretic mobility shift assay (EMSA) and confocal imaging of the fluoresceinated complex. These techniques were used to determine if there was successful non-covalent attachment between Tat₂ and a plasmid cargo. Complex formation is an essential element to CPP transformation.

3.1.1 Electrophoretic mobility shift assay

The formation of a CPP-cargo complex and the physical changes associated with the attachment of a peptide to a DNA molecule were observed using this assay. Eight ratios of CPP to plasmid were investigated (0.5:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, and 7:1), with the same amount of plasmid used in each combination. Shifts in plasmid movement can be observed in the lanes that contain both CPP and plasmid components, starting at a ratio of 1:1 (Figure 9). At ratios of 2:1 and higher, shifts of greater magnitude can be seen. These shifts are positive results for this assay, signifying the formation of a complex between Tat₂ and pBI221. The bands on the gel represent the plasmid component; all shifts therefore represent changes in the electrophoretic behavior of the plasmid. Increased gel mobility shifts were observed with larger CPP:plasmid ratios, however, shifts with ratios of 3:1 and higher are not as pronounced.

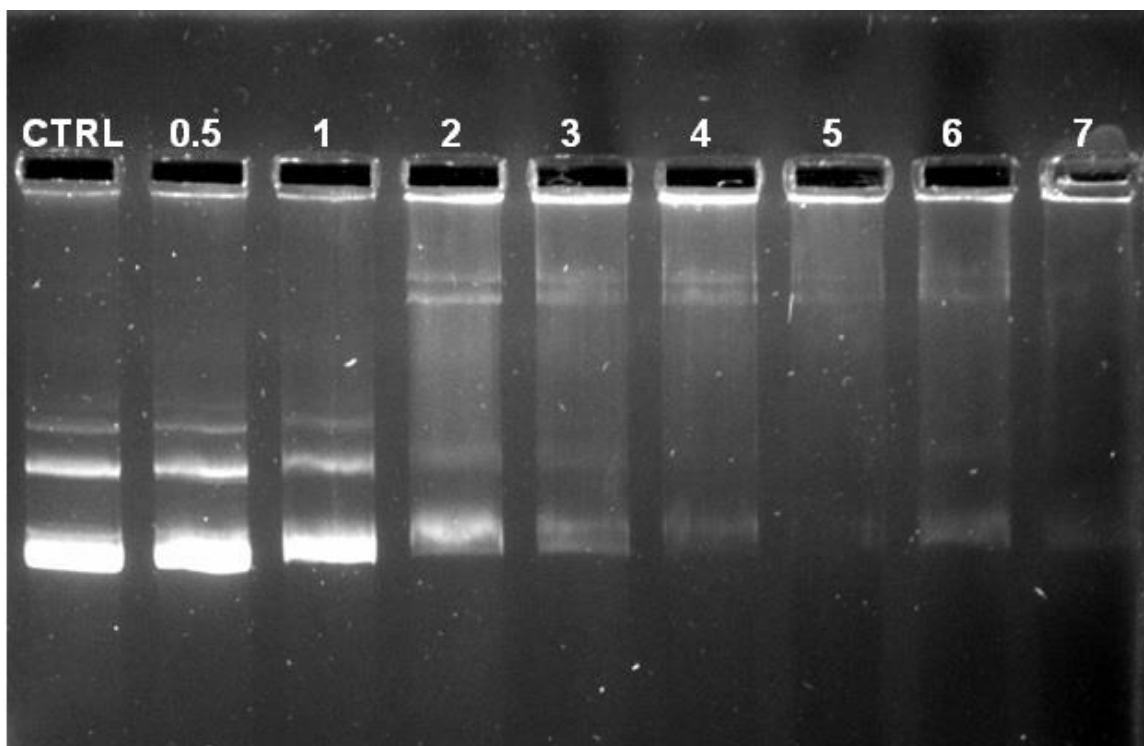


Figure 9: Electrophoretic mobility shift assay of Tat₂:pBI221 complexes. Various ratios of Tat₂ and pBI221 components were combined and run on a 0.8% agarose gel containing SYBR® Safe. The lanes are numbered with the ratio of Tat₂ to pBI221 plasmid.

During the process of plasmid isolation there are modifications to the plasmid, creating different plasmid states. Single-stranded nicks in the plasmid lead to a nicked open circular state, while double-stranded cleavages in the plasmid lead to a relaxed linear state. Each plasmid state demonstrates different electrophoretic movement in an agarose gel, with the supercoiled plasmid state migrating the farthest and the relaxed linear and nicked open circular states migrating a shorter distance very close together (González and Carlton 1980, Hightower et al. 1987). In the EMSA gel there are three bands per lane, each representing a different plasmid state. The presence of the CPP component affects the migration of all three plasmid states differently. At ratios of 2:1 and higher a doublet can be seen running much further up on the gel, this depicts the relaxed linear and nicked open circular plasmid states. These two states of the plasmid experience larger changes in their electrophoretic movement, as a result of CPP attachment, than the supercoiled state of the plasmid.

3.1.2 Confocal imaging of fluorescent complexes

The interaction between Tat₂ and pBI221 was further examined using a confocal laser scanning microscope to visualize the fluoresceinated complex. FITC-labelled Tat₂ and Alexa Fluor 647-labelled pBI221 were imaged together. Differences in the behavior of each component can be seen. Fluoresceinated Tat₂ appears to be evenly spread but displays various levels of intensity in small circles (Figure 10A), while the fluoresceinated plasmid appears as individual dots or clumps (Figure 10B); this pattern was evident in all four replicates. In the merged image (Figure 10C), the small circles of FITC Tat₂ appear to surround the plasmid clumps. This points to co-localization between FITC-Tat₂ and Alexa Fluor 647-pBI221, as well as variability in complex size; a pattern that has been previously

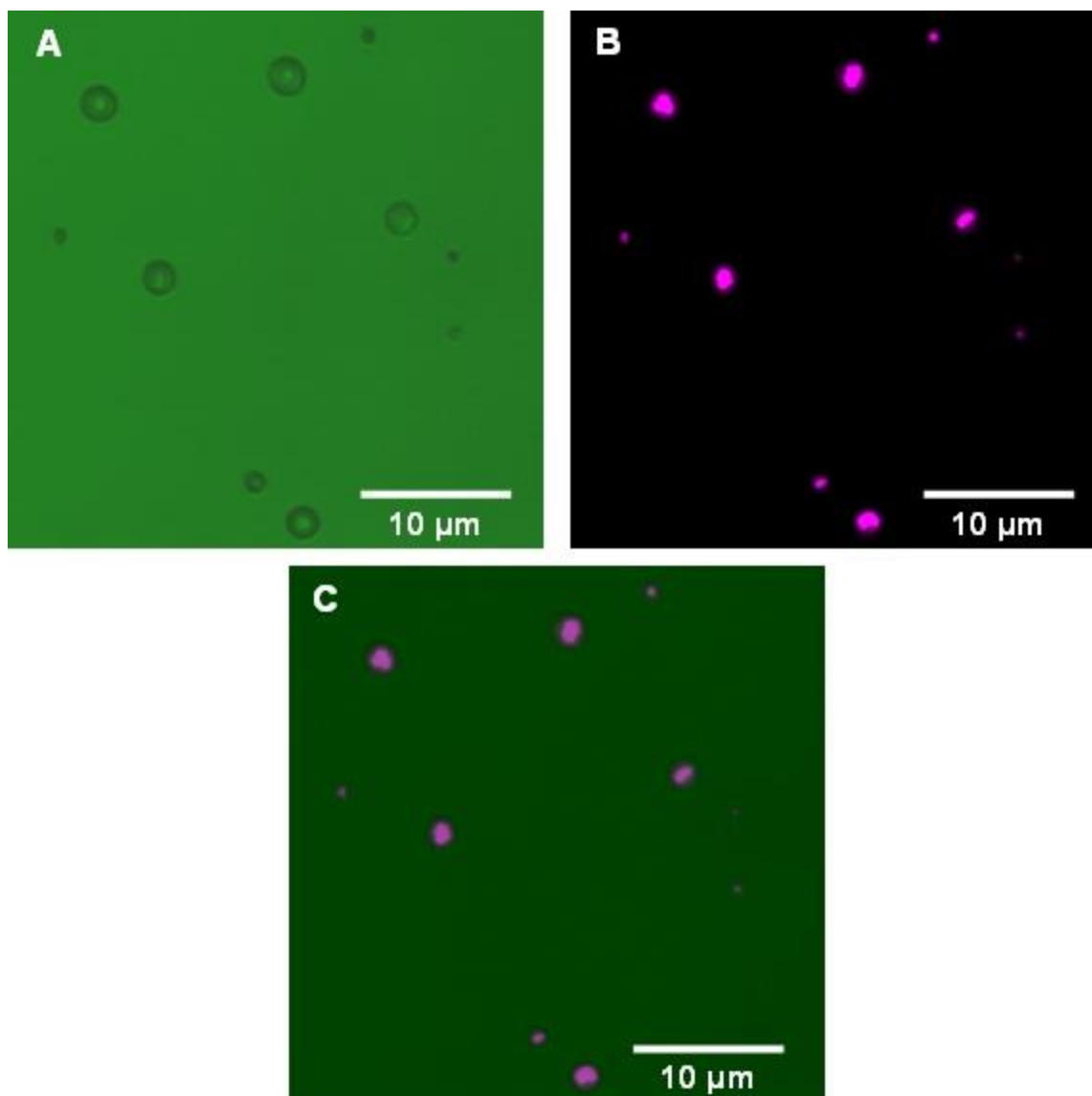


Figure 10: Complex formation between FITC-Tat₂ and Alexa Fluor 647-pBI221 in solution. Images were taken using a confocal laser scanning microscope. Fluoresceinated Tat₂ and pBI221 were combined in a 4:1 ratio and incubated for 1 h before being imaged. Complexes ranged in size from 1 to 2 µm. Colour of Alexa Fluor 647 fluorescence has been modified from blue to magenta for better visualization. A: FITC fluorescence, B: Alexa Fluor 647 fluorescence, C: merge.

observed (Chugh and Eudes 2008b). These images provide additional evidence for complex formation between Tat₂ and pBI221.

3.2 Cellular Entry of Tat₂

The ability of Tat₂ to enter unaccompanied into each cell type was investigated using FITC-labelled Tat₂ and a confocal laser scanning microscope. Cellular entry of the CPP is an important precursor to CPP transformation.

3.2.1 Soybean somatic embryo clusters

Upon incubation of labelled Tat₂ with soybean clusters, there is no evidence for entry of FITC-Tat₂ (green) into the intracellular space of soybean somatic embryo cells. The CPP appears to be localized in the intercellular space, with the bulk of the fluorescent signal being detected along the cell walls (Figure 11). These images are of soybean somatic embryo cluster tissue layers, with multiple cells in a single layer. There was no detectable fluorescent signal from negative controls of treatment with sterile water.

3.2.2 *Arabidopsis* protoplasts

The cellular entry of FITC-Tat₂ (green) into *Arabidopsis* protoplasts is shown in Figure 12. The Tat₂ fluorescence signal is strong and detected in the intracellular space of the protoplasts. Chlorophyll autofluorescence (red) can be seen inside the protoplasts as well. There is no overlap between the signal from the chlorophyll and that of the fluorescently-labelled CPP, ruling out localization of Tat₂ inside chloroplasts. On average, 69.33% of the protoplasts displayed internalization of FITC-Tat₂ (Table 1). These results evidence

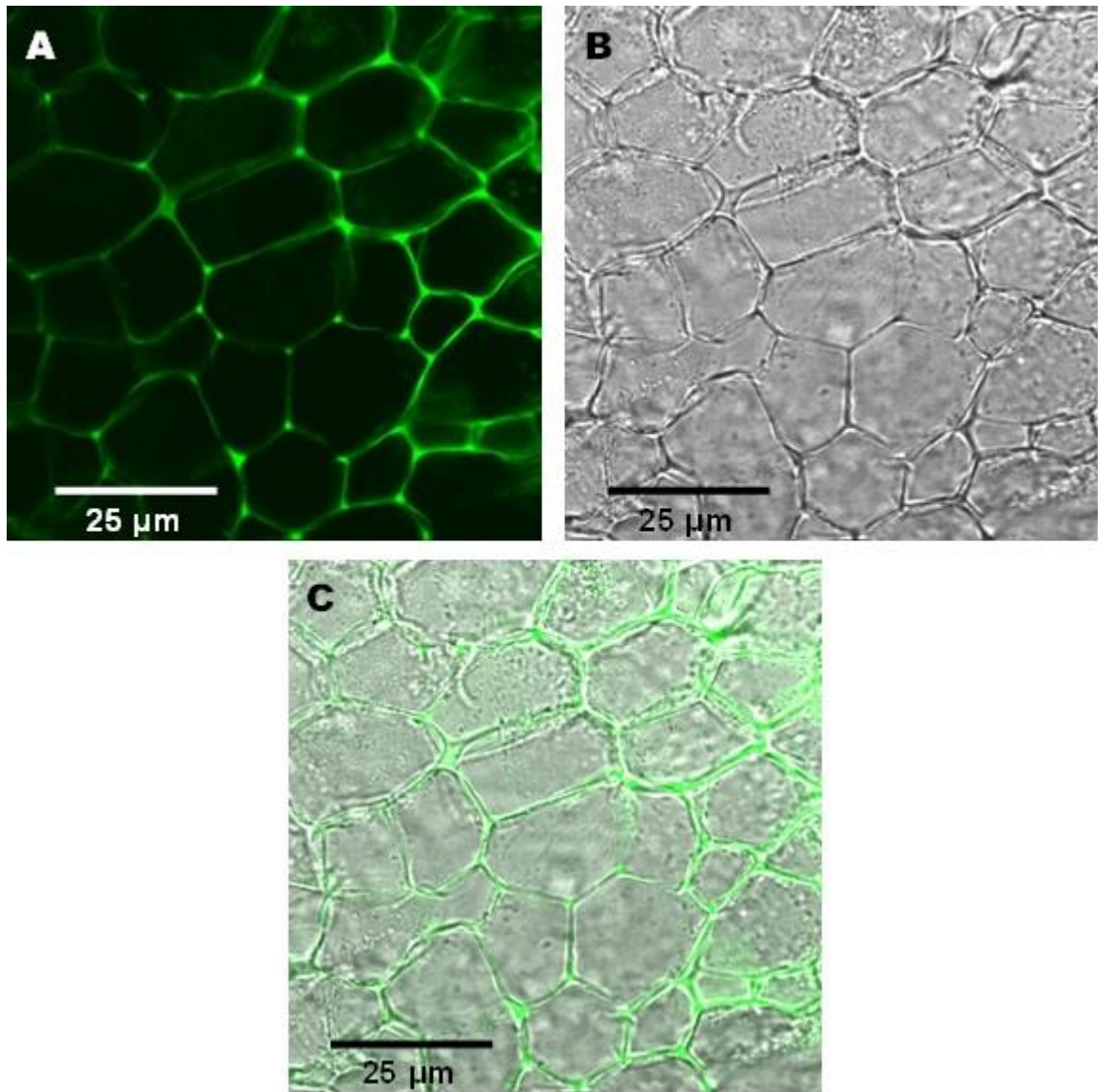


Figure 11: Fluoresceinated Tat₂ and soybean somatic embryo cells. This soybean somatic embryo cluster was treated with 4 μg of FITC-Tat₂ for 1 h, sliced into layers, and then imaged with a confocal laser scanning microscope. A: FITC fluorescence, B: brightfield, C: merged image.

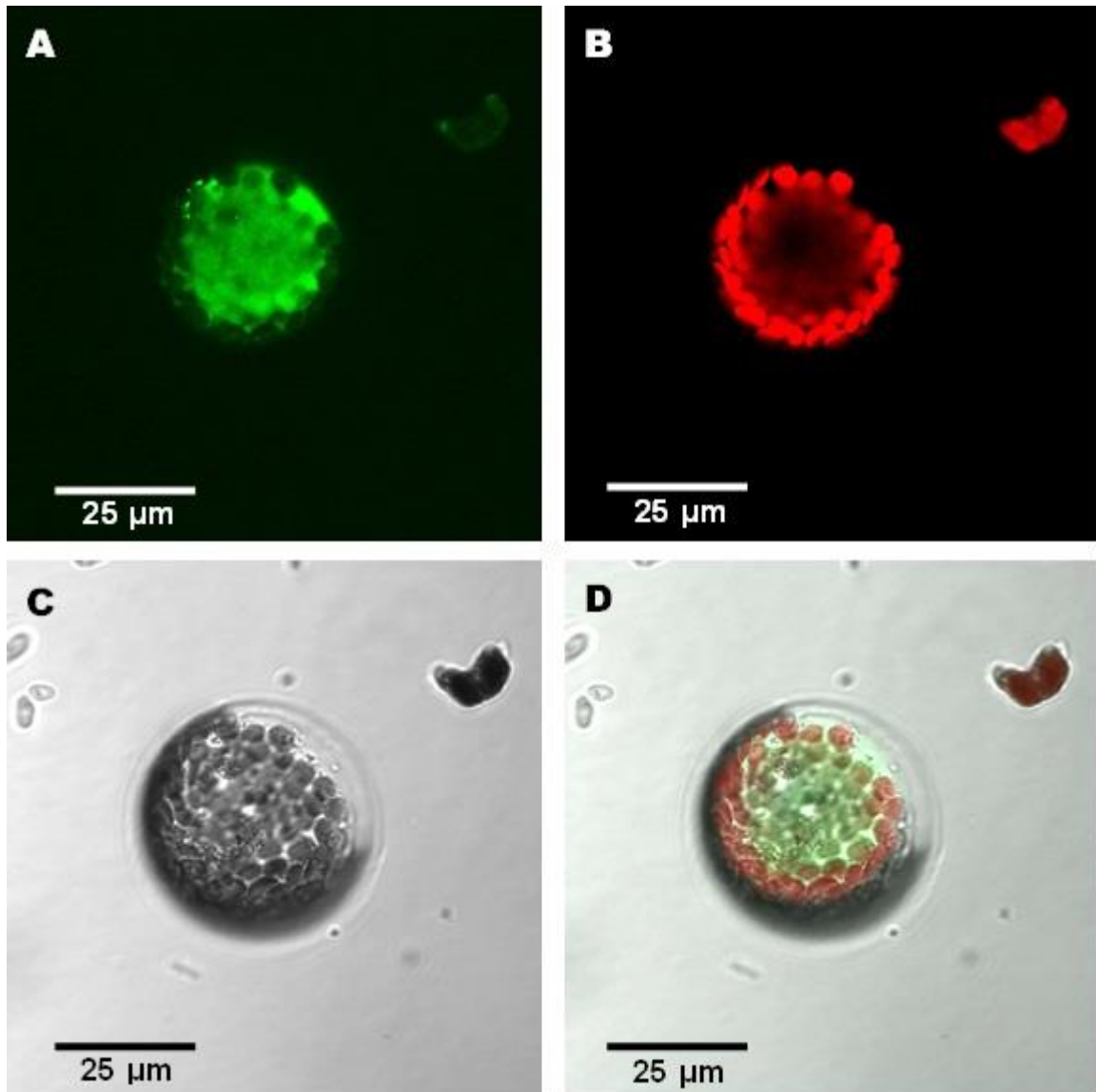


Figure 12: Uptake of fluorescently-labelled Tat₂ into an intact *Arabidopsis* protoplast. The protoplast was treated with 8 µg FITC-Tat₂ and imaged with a confocal laser scanning microscope, chlorophyll autofluorescence (red) was detected. A: FITC fluorescence, B: chlorophyll autofluorescence, C: brightfield, D: merged.

Table 1: Intracellular FITC-Tat₂ expression in *Arabidopsis* protoplasts. Protoplasts exposed to 8 μ g FITC-Tat₂ were observed under the confocal laser scanning microscope. In each experiment the proportion of protoplasts displaying FITC fluorescence was recorded (n=100 protoplasts viewed).

	Percent Expressing FITC (%)
Experiment #1	79
Experiment #2	63
Experiment #3	66
AVERAGE	69.33

the ability of Tat₂ to enter into protoplast cells. Only a chlorophyll fluorescence signal was detectable in negative controls of treatment with sterile water.

3.3 Uptake of Fluorescently-Labelled Complexes

Fluorescently-labelled complexes were created once again by combining separately fluoresceinated Tat₂ and plasmids. These fluoresceinated complexes were used to examine the entry of a CPP-cargo complex into both plant materials. The role of Tat₂ as a carrier was investigated in these experiments. Observations of the fluoresceinated complexes entry into cells were carried out using a confocal laser scanning microscope.

3.3.1 Soybean somatic embryo clusters

Tat₂ fluorescence can be observed (green) but there is no observable signal from pBI221 (Figure 13). Although there is evidence that the complexes do form, there is no signal from the plasmid component. This may be a result of the rinsing stage that occurs after incubation, where molecules that have not entered into the intracellular or intercellular space are removed. The Tat₂ signal observed does not appear to penetrate into the intracellular space but is instead localized to the intercellular space. The strength of the signal is quite similar to that of unaccompanied Tat₂, but the depth to which it penetrates into the somatic embryo tissue layers is not as deep. There was no detectable fluorescent signal from the plasmid in negative controls consisting of exposure to fluoresceinated pBI221 on its own and exposure to sterile water.

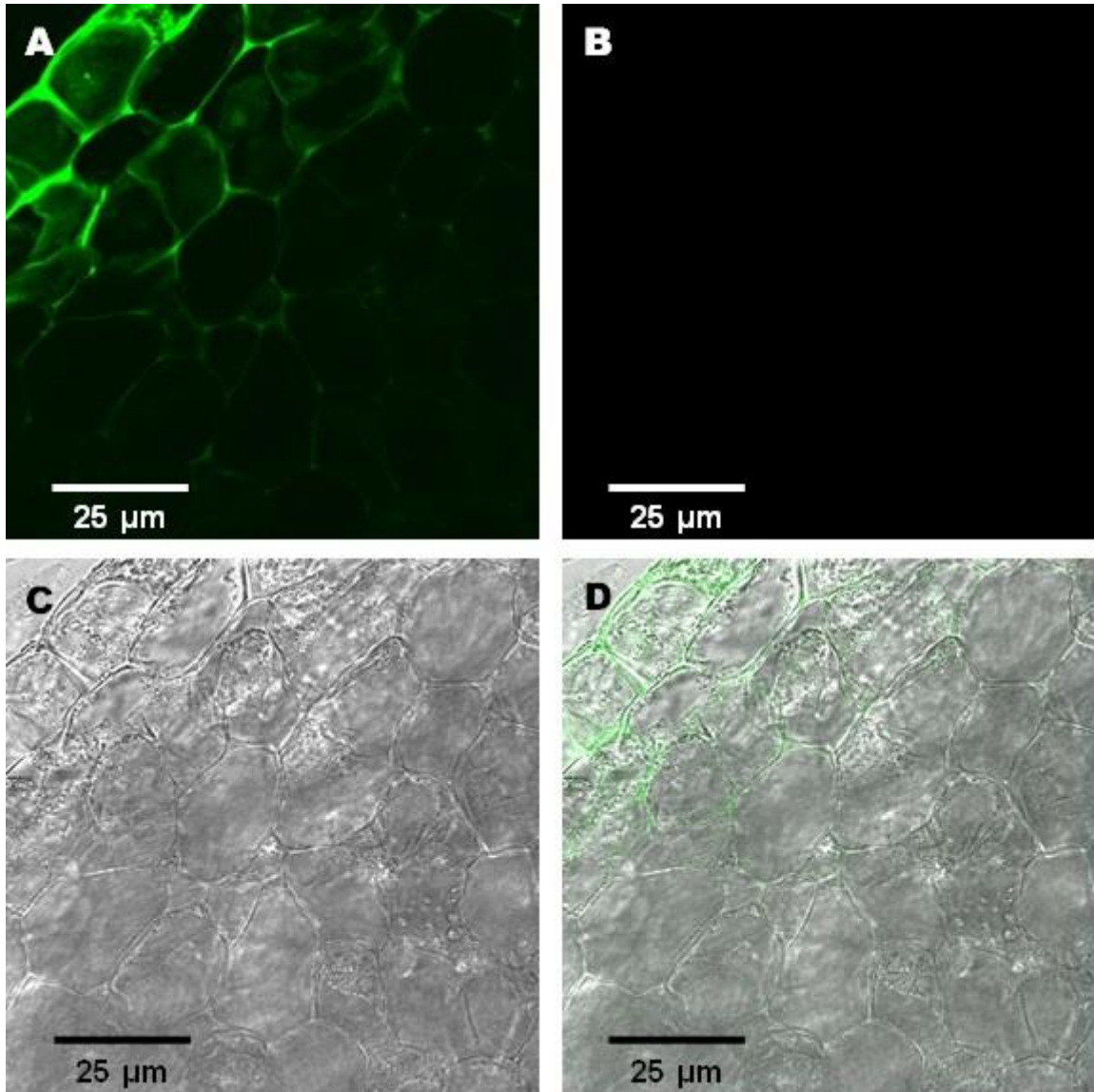


Figure 13: Fluoresceinated CPP-cargo complexes in the intercellular space between soybean somatic embryo cells. Soybean somatic embryo clusters were treated with a combination of 20 μg FITC-Tat₂ and 5 μg Alexa Fluor 647-pBI221 for 1 h and then imaged with a confocal laser scanning microscope. A: FITC fluorescence, B: Alexa Fluor 647 fluorescence, C: brightfield, D: merge.

3.3.2 *Arabidopsis* protoplasts

Uptake of the fluoresceinated complex was not observed in *Arabidopsis* protoplasts (Figure 14), as there is no detectable fluorescence signal from the plasmid component, Cy3-labelled pDRYFP. However, there is a clear signal from the CPP component, FITC-Tat₂ (green), inside the protoplasts (Table 2). This pattern may once again indicate that the fluoresceinated complexes were removed during the wash stage, due to their unsuccessful entry into the protoplasts. Chlorophyll autofluorescence (red) is also visible and does not overlap with the FITC-Tat₂ signal. Negative controls of treatment with Cy3-pDRYFP and treatment with sterile water did not yield fluorescent signals for the plasmid.

3.4 Tat₂-Mediated Transformation

The use of Tat₂ as a transformation method was investigated in soybean somatic embryo clusters and *Arabidopsis* protoplasts. The results from the previous set of experiments indicated that there was no uptake of fluoresceinated complexes in either tissue system. The fluorescent tags on each of the components may have inhibited complex formation or complex penetration into the cells. These Tat₂-mediated transformation experiments were carried out to observe the entry of unlabeled CPP-cargo complexes into these tissue systems. Transformation efficiency was observed by assaying the transient expression of the respective reporter genes.

3.4.1 Soybean somatic embryo clusters

Embryo clusters were treated with Tat₂-pBI221 complexes and assayed for GUS expression using a GUS histochemical assay. Each embryo cluster was individually

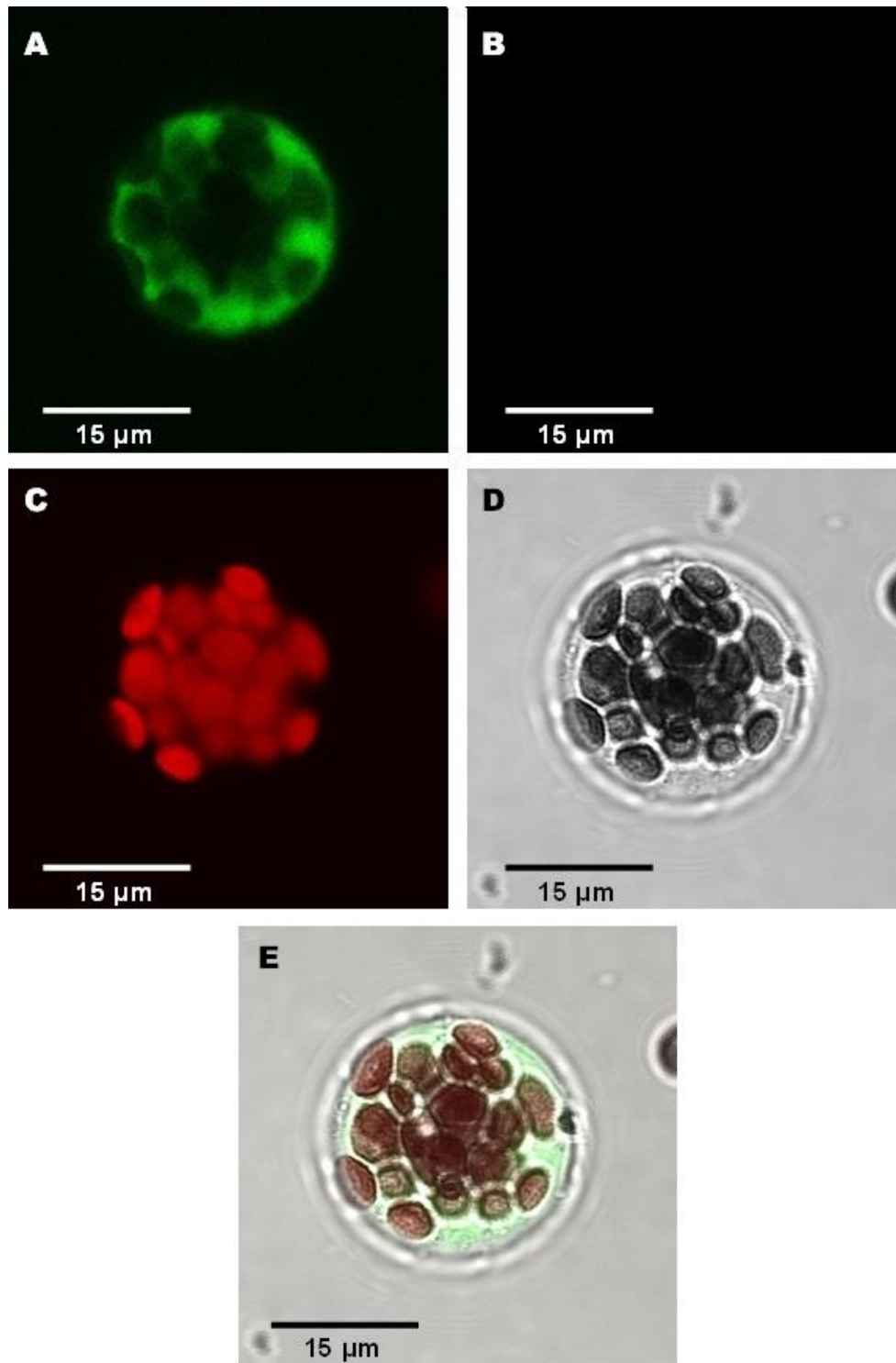


Figure 14: Uptake of fluoresceinated CPP-cargo complex components in an *Arabidopsis* protoplast. The protoplast was exposed to a combination of 20 μg FITC-Tat₂ and 5 μg Cy3-pDRYFP and then imaged with a confocal laser scanning microscope, chlorophyll autofluorescence was detected. A: FITC fluorescence, B: Cy3 fluorescence, C: chlorophyll autofluorescence, D: brightfield, E: merge.

Table 2: Intracellular FITC and Cy3 fluorescence in *Arabidopsis* protoplasts. Protoplasts were exposed to fluoresceinated CPP-cargo complexes consisting of 5 μ g FITC-Tat₂ and 20 μ g Cy3-pBI221. In each experiment the proportion of protoplasts displaying FITC and Cy3 fluorescence was recorded (n=100 protoplasts viewed).

	Percent Expressing FITC (%)	Percent Expressing Cy3 (%)
Experiment #1	68	0
Experiment #2	61	0
Experiment #3	48	0
AVERAGE	59	0

inspected for the appearance of any positive staining, indicated by blue pigmentation. No positive reactions to the GUS stain were observed. In all instances, regardless of incubation time, temperature, or the amounts of Tat₂ and pBI221, there were no successful GUS histochemical stains post-transformation. Negative controls, including exposure to plasmid alone and sterile water, also yielded negative responses to the GUS stain.

Particle bombardment with pBI221 was also used to transform the soybean somatic embryo clusters. In each replicate there were clusters displaying a positive response to the GUS stain (Figure 15). The average rate of successful GUS staining using this method was 46.76% of the soybean somatic embryo clusters (Table 3), with between 1 and 30 individual blue spots observed on each cluster.

3.4.2 *Arabidopsis* protoplasts

Arabidopsis protoplasts treated with Tat₂-pDRYFP complexes were evaluated under the confocal laser scanning microscope to assay transient expression of YFP. Confocal images for both the lower (Figure 16) and higher (Figure 17) levels of CPP-plasmid complexes did not yield a detectable YFP signal. Negative controls involving treatment with the plasmid alone and sterile water also did not yield any signal.

PEG-mediated transformation with pDRYFP was also carried out. Successful transformations were observed using this method, with a detectable membrane-localized signal (Figure 18). On average, 10.25% of these protoplasts responded positively to this type of transformation (Table 4).



Figure 15: Soybean somatic embryo cluster displaying positive GUS stain after particle bombardment. This cluster underwent particle bombardment with pBI221 and was subjected to a GUS stain three days post-transformation. After an overnight incubation and destaining the cluster was imaged.

Table 3: GUS expression in soybean somatic embryo clusters following particle bombardment. Clusters were bombarded with pBI221 and those displaying staining were evaluated.

	Number of Embryo Clusters	Number Expressing GUS	Percent Stained (%)
Experiment #1	176	97	55.11
Experiment #2	174	86	49.43
Experiment #3	193	69	35.75
AVERAGE	181	84	46.76

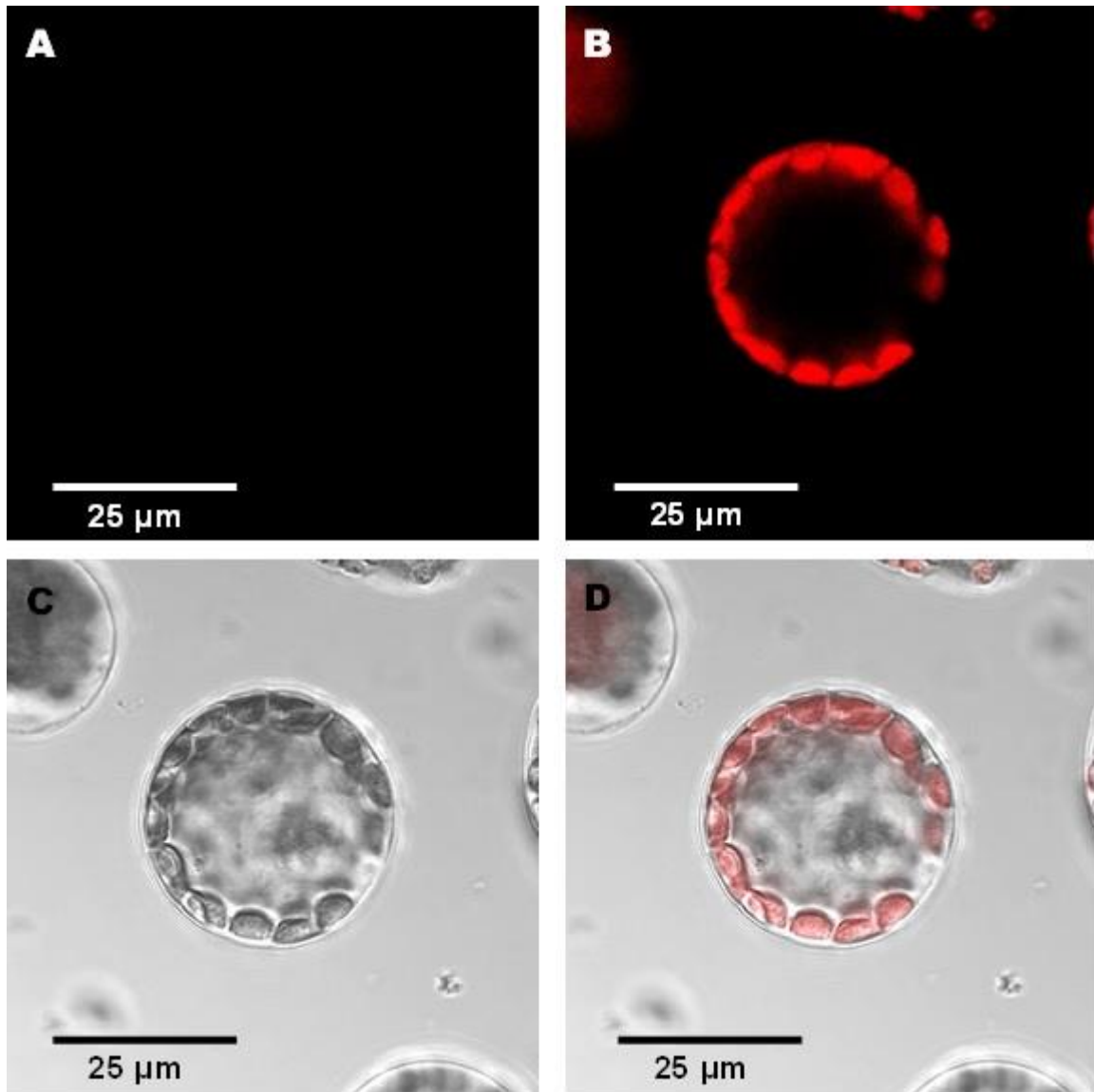


Figure 16: *Arabidopsis* protoplast after low level CPP-mediated transformation. This protoplast was treated with 8 μg of Tat₂ and 2 μg of pDRYFP. Images were taken with a confocal laser scanning microscope after 1 h incubation, chlorophyll autofluorescence was detected. A: YFP fluorescence, B: chlorophyll autofluorescence, C: brightfield, D: merge.

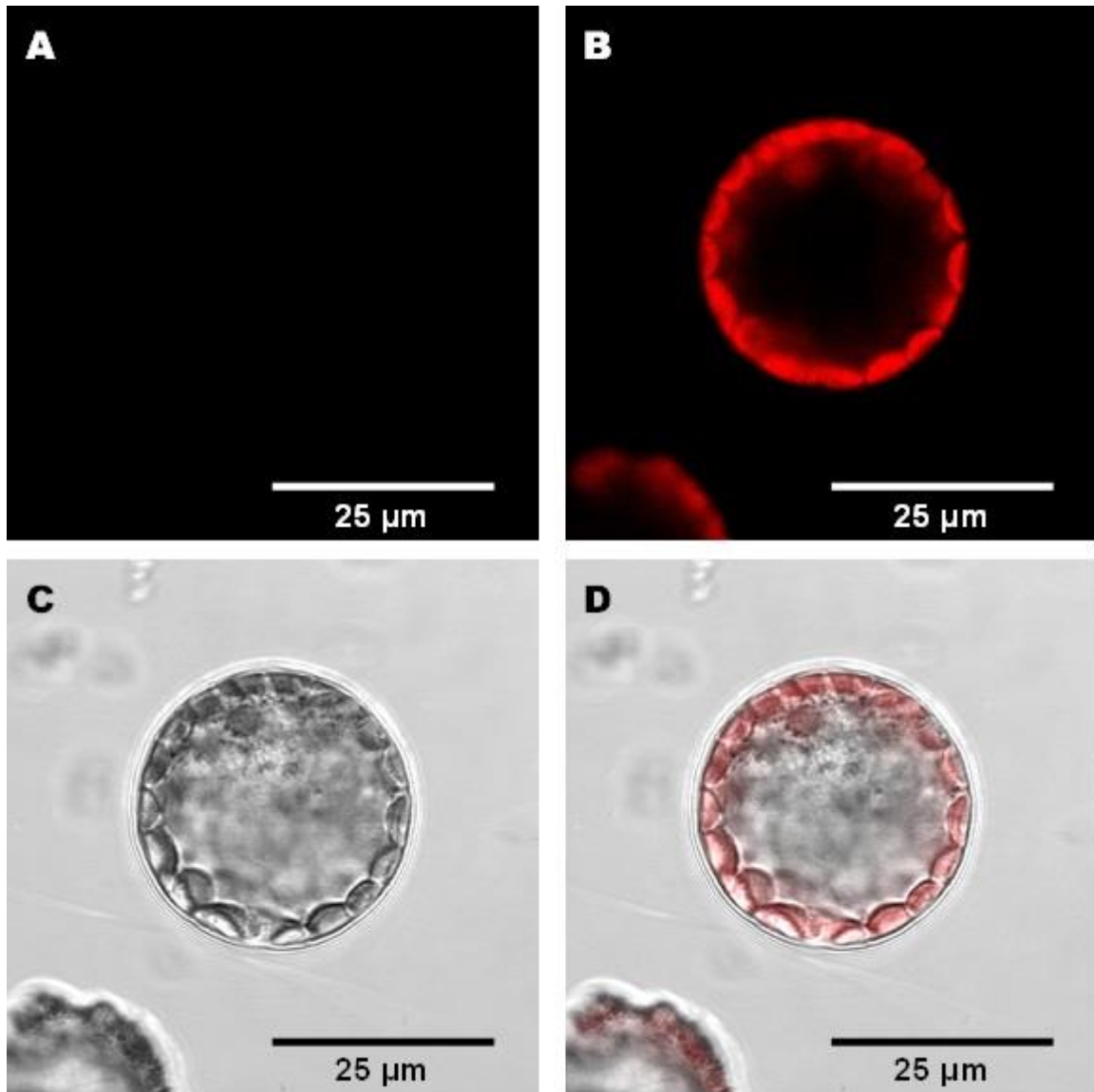


Figure 17: *Arabidopsis* protoplast after high level CPP-mediated transformation. This protoplast was treated with 80 μg of Tat₂ and 20 μg of pDRYFP. Images were taken with a confocal laser scanning microscope after 1 h incubation, chlorophyll autofluorescence was detected. A: YFP fluorescence, B: chlorophyll autofluorescence, C: brightfield, D: merge.

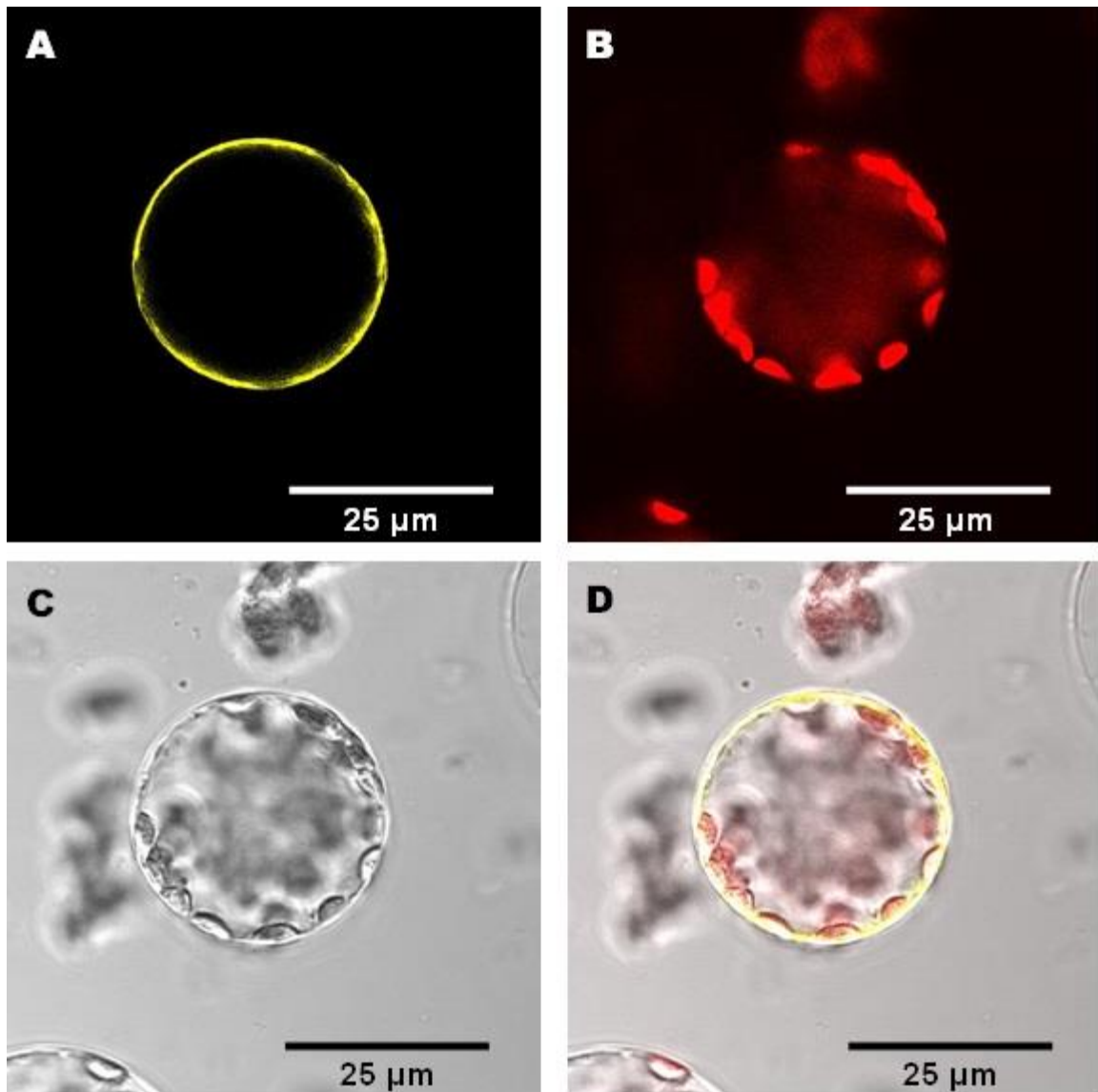


Figure 18: PEG calcium-mediated transformation of *Arabidopsis* protoplast. This protoplast was transformed with 20 µg of pDRYFP using PEG calcium-mediated transformation. After an overnight incubation it was then imaged using a confocal laser scanning microscope, chlorophyll autofluorescence was detected. A: YFP fluorescence, B: chlorophyll autofluorescence, C: brightfield, D: merge.

Table 4: PEG calcium-mediated transformations of *Arabidopsis* protoplasts. Protoplasts were transformed with pDRYFP and those with YFP fluorescence were evaluated.

	Number of Protoplasts	Number Expressing YFP	Percent Expressing YFP (%)
Experiment #1	100	7	7
Experiment #2	100	5	5
Experiment #3	100	5	5
Experiment #4	300	38	12.67
Experiment #5	300	32	10.67
Experiment #6	300	36	12
AVERAGE	200	20.5	10.25

4 Discussion

With the availability of fully sequenced plant genomes there is more opportunity than ever to realize the potential of plant trait genetic modification. Traits important to agronomic growth, maturation, crop yield, and flowering can now be identified and manipulated (Bolger et al. 2014). Current plant transformation techniques can be used quite successfully in certain systems. However, the efficiency of said techniques differs across species, genotypes, and tissues (De Cleene and De Ley 1976, De Cleene 1985, Cheng et al. 2004). Novel plant transformation techniques are being developed to increase the efficiency of plant transformation, as well as the range of plants and tissues that can be transformed. Cell-penetrating peptide transformation is one such proposed technique. In animal systems, CPP transformation is quite developed, and has a wide demonstrated range of applicability (Sawant et al. 2006, Yang et al. 2014). The hope is to achieve the same level of success for CPP transformation in plants.

In this study the prospect of CPP transformation for use in two tissue culture systems, soybean somatic embryos and *Arabidopsis* protoplasts, was investigated. Each system was selected to test transformation in regenerable cells of an important species; *Arabidopsis* as a key model species and soybean as a major crop of economic value. The benefit to the use of these systems is that the explants used can regenerate into a whole organism (Li et al. 1985, Chupeau et al. 2013). Transformations carried out on regenerable tissues therefore have the potential to become a successfully transformed mature plant, which has implications for stable transformations and the inheritance of altered genes. Soybean somatic embryos and *Arabidopsis* protoplasts have both been previously transformed using

other transformation methods (Finer and McMullen 1991, Yoo et al. 2007). Their demonstrated amenability to genetic transformation contributed to their selection as the materials to be studied. As well, there was no previous history of CPP-mediated transformation to achieve transient expression in these specific systems.

There are a few major steps required for successful CPP-mediated transformation. The formation of a complex between the CPP and the cargo must occur before the cargo can translocate into the cell with the CPP. The CPP used in this study, Tat₂, is capable of forming complexes with a variety of cargo molecules. In the current study, complex formation between Tat₂ and pBI221 was observed using an electrophoretic mobility shift assay (Figure 9) and confocal microscopy images of the fluoresceinated complex (Figure 10). Both sets of results were positive and provided two sets of evidence indicating Tat₂ can form a complex with pBI221. In the EMSA results, binding of the two components was observed. The distance the plasmid migrated on the gel was different from the distance the plasmid migrated when combined with the CPP at ratios of 1:1 and higher. This shift means that Tat₂ attached to the DNA and that the newly formed complex possesses a different charge and weight than the unbound DNA (Garner and Revzin 1981). The bandshifts in the gel may therefore represent conformational changes associated with CPP-plasmid complex formation.

The confocal images of the FITC-Tat₂ and Alexa Fluor 647-pBI221 complex serve two purposes. The first is demonstrating that the labelling of both components was successful. The second is providing a visual representation of the relationship between the CPP and plasmid. The results from the EMSA indicate that there is attachment between the two components. In the confocal images (Figure 10), the components and their interaction can

be visualized, further confirming complex formation. The formation of a complex using Tat₂ with a different plasmid cargo has been previously reported, where the CPP has also been shown to serve a protective function (Chugh and Eudes 2008b). Confirmation of Tat₂'s ability to form a complex with a plasmid cargo molecule means that the first step of CPP-mediated transformation is successful.

The cellular entry of the CPP is essential to the use of CPPs as transporters. The CPP must be capable of entering the cell on its own before it can carry along a cargo molecule. The purpose of viewing the fluoresceinated CPP's movement was to determine the extent to which it is capable of penetrating both cell types examined. FITC-Tat₂ does not appear to enter into soybean somatic embryo cluster cells (Figure 11). This may be due in part to the presence of a cell wall. As previously mentioned, CPP work in animals is decades ahead of CPP work in plants. There are a number of differences between plant and animal cells, one of the biggest being that animal cells do not possess a cell wall. Soybean somatic embryo cells have a completely intact and fully developed cell wall, which may constrain the movement of the CPP into the cell. Additionally, in the confocal images taken there is no way to clearly view single cells. Even in the layers the embryo clusters have been sliced into, there is overlap and multiple cells per frame. In some cases it appears as though there may be some fluoresceinated Tat₂ within the cells themselves, but this cannot be confirmed solely based on the images presented here. A more detailed look into the uptake of fluoresceinated Tat₂ into soybean somatic embryo cluster cells is therefore needed.

Arabidopsis protoplasts have had their cell wall removed, allowing them to share more similarity with animal cells than the soybean somatic embryo cluster cells. The uptake of FITC-Tat₂ was viewable inside individual *Arabidopsis* protoplasts (Figure 12). As a result,

the translocation of FITC-Tat₂ was better observed in this system than in the soybean somatic embryo cluster cells. The movement of the CPP within the protoplasts does not appear to be localized to a single point. Tat₂ has previously been reported to possess a nuclear localization signal (Chugh and Eudes 2007), however, in these images the fluoresceinated Tat₂ does not seem to have any limits to where it can move within the cell, with the exception of inside chloroplasts. Despite the widespread movement of the CPP inside the protoplast, the actual uptake of the CPP only occurs in about two-thirds of the protoplasts examined. This means that there are additional factors that make certain protoplasts viable for CPP penetration while others are not. As well, the movement of FITC-Tat₂ in relation to both of the cell systems examined may not solely be related to the presence or lack of a cell wall.

Entry of the CPP-cargo complex into the cell is another step required for CPP-mediated transformation. The CPP-cargo complex must be capable of moving into the cell in order to reach the nucleus and have the cargo expressed. Observations of a fluoresceinated complex, made up of FITC-Tat₂ and Alexa Fluor 647-pBI221, into soybean somatic embryo cluster cells (Figure 13) were similar to those of fluorescently-labelled Tat₂ on its own (Figure 11). In both instances there is visible FITC-Tat₂ in the intercellular space of soybean somatic embryo cluster cells, and there once again does not appear to be any uptake of the fluoresceinated CPP into the intracellular space. There is a lack of a signal from fluorescently-labelled pBI221, which indicates that there is no movement of pBI221 into the cells, either on its own or in a complex with fluoresceinated Tat₂.

The uptake of a fluoresceinated CPP-cargo complex, consisting of FITC-Tat₂ and Cy3-pDRYFP, was also not observed in *Arabidopsis* protoplasts (Figure 14). In the previous

set of experiments, a lack of cell wall seemed to give the protoplasts an advantage in the uptake of FITC-Tat₂. However, the lack of a cell wall does not seem to provide the protoplasts with any advantage when it comes to the uptake of a fluoresceinated complex. It appears that the protoplasts' plasma membrane does not allow entry of CPPs once they are attached to cargo. There is no detectable signal from the fluoresceinated plasmid and therefore no associated uptake of the plasmid into the protoplast. The signal from FITC-Tat₂ in this experiment is very close in strength and quantifiable uptake to the signal observed in the experiments with the CPP on its own (Figure 12), this would imply that only unbound CPP entered into the cells.

The trends observed for fluoresceinated complex uptake are very similar to those observed in fluoresceinated CPP uptake. In both cases, the movement of FITC-Tat₂ into the intercellular space of soybean somatic embryo cells and the intracellular space of *Arabidopsis* protoplasts was observed. Images from experiments where the plant materials were exposed to fluorescently-labelled CPP are almost identical to images from experiments where the plant materials were exposed to fluorescently-labelled complexes. The absence of a signal from the plasmid and the presence of a signal from the CPP indicate that there was no uptake of complexed CPP and plasmid. This may mean that although the CPP can form complexes with the cargo, there is no CPP-mediated uptake of these complexes into the plant materials examined. It appears that excess unbound CPP from the CPP-cargo complex solutions is able to enter into the plant materials in the same way that it does when the CPP is applied on its own. The absence of fluoresceinated complexes in confocal images for both soybean somatic embryo clusters and *Arabidopsis* protoplasts is most likely attributable to the wash and rinse stages during treatment. These stages serve

to remove all unbound molecules from the plant materials before they are imaged, and are essential to accurate observations of CPP and complex uptake (Vivès et al. 1997, Kaplan et al. 2005).

The final set of experiments involved carrying out CPP-mediated transformation in both systems to examine the entry of unlabeled CPP and plasmid components. This process involves all stages investigated in the previous experiments working together to achieve transient expression of the reporter genes contained within each plasmid. Soybean somatic embryo clusters were subjected to CPP-mediated transformations under various parameters. These parameters were investigated in the hopes of determining which factors might have an effect on CPP transformation in this system. Incubation time, temperature, differing CPP-plasmid ratios and component amounts were all investigated. All replicates and experiments under the parameters in this study did not yield successful transient expression of GUS.

Particle bombardment of soybean somatic embryo clusters was included as a positive control because of its documented ability to achieve transient expression in this tissue type (Finer and McMullen 1991). The bombardments were carried out using soybean somatic embryo clusters and pBI221, making the mode of transformation the central difference between the bombardments and the CPP transformations. This control was used to determine if the negative results of the CPP transformations were a consequence of issues with the plasmid or plant material used. The positive GUS staining resulting from bombardments with pBI221 confirm that this plasmid contains genes for GUS, and that successful transformation will lead to GUS expression detectable through histochemical staining (Figure 15). The lack of positive GUS staining in the CPP transformation

experiments was therefore a result of unsuccessful transformation of the plasmid, and not a problem with the plasmid itself. The positive results of the bombardments also point to the amenability of soybean somatic embryo clusters to transformation, indicating that the clusters did not present an issue in the CPP transformations.

CPP-mediated transformation was investigated in *Arabidopsis* protoplasts using pDRYFP. Two sets of these transformations were carried out, one involving low levels of CPP and plasmid and the other involving high levels of CPP and plasmid. The low level transformations were used to keep the amount of liquid to a minimum and test for the use of a lower amount of plasmid. The osmotic potential for protoplasts has a great effect on protoplast viability (Cocking 1972); therefore using small amounts of liquid can increase protoplast recovery after transformation. The high level transformation was used to mimic the amount of plasmid involved in PEG-mediated transformations, the primary mode of protoplast transformation. However, the amount of liquid required was five times higher than the amount used in the low level transformations. In each of the experiments carried out there was no detectable YFP signal (Figure 16, Figure 17). Once again a positive control was carried out, using the same plant material and plasmid. PEG calcium-mediated transformations of the *Arabidopsis* protoplasts were successful, conferring YFP expression in the cellular membranes (Figure 18). The results from this positive control point to no issues with the use of pDRYFP or with the protoplasts themselves.

A number of things have been demonstrated here about Tat₂ and Tat₂-mediated transformation in *Arabidopsis* protoplasts and soybean somatic embryo clusters. The ability of Tat₂ to form non-covalent complexes with a plasmid cargo has been confirmed. Fluorescently-labelled Tat₂ and fluorescently-labelled Tat₂-plasmid complex uptake does

not seem to be viable in soybean somatic embryo clusters, possibly due to the presence of a cell wall. However, there appears to be potential for further work on these areas in *Arabidopsis* protoplasts. It follows that from the results of the complex uptake experiments there wasn't any success in the CPP-mediated transformations for both systems. In *Arabidopsis* protoplasts the issue seems to be in the ability of Tat₂ to enter into the cells carrying a plasmid cargo molecule. In soybean somatic embryo clusters, the translocation of unaccompanied Tat₂ into the cells is the first obstacle that must be overcome before transformation using this CPP can occur.

The results presented are not to be taken as a representation of all CPP-mediated transformations in plants. The scope of this study is limited to Tat₂-mediated transformations of two tissue culture systems under the parameters investigated. The mechanisms for CPP-mediated transformations have not yet been ascertained (Copolovici et al. 2014). As such, it is difficult to determine where the faults may lie in unsuccessful transformations. The experiments in this study helped to narrow down the problematic stages of Tat₂-mediated transformations in *Arabidopsis* protoplasts and soybean somatic embryo clusters. Nevertheless, within each of these steps there remains a wide array of possibilities as to the exact cause of the problem. In this study, fluoresceinated components were used to help examine the movement of CPP and plasmid components. The fluorescent labelling process, however, will have resulted in a conformational change of each product. This may contribute to a discrepancy between what was observed in this study and what actually occurs when the unlabeled components interact with the cells and each other. Additionally, the focus of this study was on the transformation and movement

of the plasmids pBI221 and pDRYFP. The use of other plasmids with different sizes and attributes, as well as linearized plasmids, may lead to a completely different set of results.

The use of CPP-mediated transformation has garnered a lot of interest in genetic transformation, especially concerning its application in plants. The hope is that this technology could become a novel plant transformation method, which may in turn increase the number of plant systems that can be genetically modified. The use of this type of transformation in plants is very new though and its successful use has only been documented in a few systems (Mäe et al. 2005, Chen et al. 2007, Chugh and Eudes 2008b, Chugh et al. 2009). The goal of this study was to contribute to the development of CPP transformation by investigating its use in two regenerable tissue culture systems. Despite the CPP transformations in both tissues being unsuccessful, information generated from these experiments can be used to guide future work in this area. Future studies may benefit from focusing on CPP-mediated transformations of protoplasts over work on tissue systems that possess cell walls. The results of this study, however, do not support further investigation of Tat₂ in protoplasts under the parameters tested. In conclusion, there remains a lot of work to be done in understanding CPP-mediated plant transformation and the range of its application.

References

- Bolger ME, Weisshaar B, Scholz U, Stein N, Usadel B, Mayer KFX. 2014. Plant genome sequencing – applications for crop improvement. *Curr Opin Biotech.* 26:31-37.
- Brasseur R, Divita G. 2010. Happy birthday cell penetrating peptides: already 20 years. *Biochim Biophys Acta.* 1798(12):2177-2181.
- Buchheim JA, Colburn SM, Ranch JP. 1989. Maturation of soybean somatic embryos and the transition to plantlet growth. *Plant Physiol.* 89(3):768-775.
- Carpita N, Vergara C. 1998. A recipe for cellulose. *Science.* 279(5351):672-673.
- Chambers R, Höfler K. 1931. Micrurgical studies on the tonoplast of *Allium cepa*. *Protoplasma.* 12:338-355.
- Chen CP, Chou JC, Liu BR, Chang M, Lee HJ. 2007. Transfection and expression of plasmid DNA in plant cells by an arginine-rich intracellular delivery peptide without protoplast preparation. *FEBS Lett.* 581(9):1891-1897.
- Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL. 2004. Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro Cell Dev-Pl.* 40:31-45.
- Chilton MD, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell.* 11(2):263-271.
- Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J. 1996. Engineered GFP as a vital reporter in plants. *Curr Biol.* 6(3):325-330.
- Christou P. 1995. Strategies for variety-independent genetic-transformation of important cereals, legumes and woody species utilizing particle bombardment. *Euphytica.* 85:13-27.
- Christou P, McCabe DE, Swain WF. 1988. Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiol.* 87(3):671-674.
- Chugh A, Amundsen E, Eudes F. 2009. Translocation of cell-penetrating peptides and delivery of their cargoes into triticale microspores. *Plant Cell Rep.* 28(5):801-810.
- Chugh A, Eudes F. 2007. Translocation and nuclear accumulation of monomer and dimer of HIV-1 Tat basic domain in triticale mesophyll protoplasts. *BBA-Biomembranes.* 1768(3):419-426.
- Chugh A, Eudes F. 2008a. Cellular uptake of cell-penetrating peptides *pVEC* and transport in plants. *J Pept Sci.* 14(4):477-481.
- Chugh A, Eudes F. 2008b. Study of uptake of cell penetrating peptides and their cargoes in permeabilized wheat immature embryos. *FEBS J.* 275(10):2403-2414.
- Chugh A, Eudes F, Shim YS. 2010. Cell-penetrating peptides: nanocarrier for macromolecule delivery in living cells. *IUBMB Life.* 62(3):183-193.
- Chupeau MC, Granier F, Pichon O, Renou JP, Gaudin V, Chupeau Y. 2013. Characterization of the early events leading to totipotency in an *Arabidopsis*

- protoplast liquid culture by temporal transcript profiling. *Plant Cell*. 25(7):2444-2463.
- Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*. 16(6):735-743.
- Cocking EC. 1960. A method for the isolation of plant protoplasts and vacuoles. *Nature*. 187:962-963.
- Cocking EC. 1972. Plant cell protoplasts – isolation and development. *Ann Rev Plant Physiol*. 23:29-50.
- Copolovici DM, Langel K, Eriste E, Langel U. 2014. Cell-penetrating peptides: design, synthesis, and applications. *ACS Nano*. 8(3):1972-1994.
- Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C. 2001. Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Mol Breeding*. 7:25-33.
- Davis SJ, Vierstra RD. 1998. Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol Biol*. 36(4):521-528.
- De Cleene M. 1985. The susceptibility of monocotyledons to *Agrobacterium tumefaciens*. *J Phytopathol*. 113:81-89.
- De Cleene M, De Ley J. 1976. The host range of crown gall. *Bot Rev*. 42(4):389-466.
- de Ruijter NCA, Verhees J, van Leeuwen W, van der Krol AR. 2003. Evaluation and comparison of the GUS, LUC and GFP reporter system for gene expression studies in plants. *Plant Biol*. 5(2):103-115.
- Derossi D, Joliot AH, Chassaing G, Prochiantz A. 1994. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem*. 269:10444-10450.
- Derossi D, Chassaing G, Prochiantz A. 1998. Trojan peptides: the penetratin system for intracellular delivery. *Trends Cell Biol*. 8(2):84-87.
- El-Shemy HA, Teraishi M, Khalafalla MM, Katsube-Tanaka T, Utsumi S, Ishimoto M. 2004. Isolation of soybean plants with stable transgene expression by visual selection based on green fluorescent protein. *Mol Breeding*. 14(3):227-238.
- Ermel FF, Follet-Gueye ML, Cibert C, Vian B, Morvan C, Catesson AM, Goldberg R. 2000. Differential localization of arabinan and galactan side chains of rhamnogalacturonan 1 in cambial derivatives. *Planta*. 210(5):732-740.
- Farrell LB, Beachy RN. 1990. Manipulation of β -glucuronidase for use as a reporter in vacuolar targeting studies. *Plant Mol Biol*. 15(6):821-825.
- Farrera-Sinfreu J, Giralt E, Castel S, Albericio F, Royo M. 2005. Cell-penetrating *cis*- γ -amino-L-proline-derived peptides. *J Am Chem Soc*. 127(26):9459-9468.
- Finer JJ, McMullen MD. 1991. Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. *In Vitro Cell Dev-Pl*. 27(4):175-182.

- Fittipaldi A, Ferrari A, Zoppé M, Arcangeli C, Pellegrini V, Beltram F, Giacca M. 2003. Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J Biol Chem.* 278:34141-34149.
- Fonseca SB, Pereira MP, Kelley SO. 2009. Recent advances in the use of cell-penetrating peptides for medical and biological applications. *Adv Drug Delivery Rev.* 61(11):953-964.
- Frankel AD, Pabo CO. 1988. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell.* 55(6):1189-1193.
- Garner MM, Revzin A. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. *Nucleic Acids Res.* 9(13):3047-3060.
- Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol Mol Biol R.* 67:16-37.
- González JM, Carlton BC. 1980. Patterns of plasmid DNA in crystalliferous and acrySTALLIFEROUS strains of *Bacillus thuringiensis*. *Plasmid.* 3:92-98.
- Groot RS, Wilson MA, Boumans RMJ. 2002. A typology for the classification, description and valuation of ecosystem functions, goods and services. *Ecol Econ.* 41(3):393-408.
- Halford NG. 2003. Genetically modified crops. London (UK): Imperial College Press.
- Hartley JL, Temple GF, Brasch MA. 2000. DNA cloning using in vitro site-specific recombination. *Genome Res.* 10:1788-1795.
- Hayashimoto A, Li Z, Murai N. 1990. A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. *Plant Physiol.* 93(3):857-863.
- Hernalsteens JP, Van Vliet F, De Beuckeleer M, Depicker A, Engler G, Lemmers M, Holsters M, Van Montagu M, Schell J. 1980. The *Agrobacterium tumefaciens* Ti plasmid as a host vector system for introducing foreign DNA in plant cells. *Nature.* 287:654-656.
- Hiei I, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6(2):271-282.
- Hightower RC, Metge DW, Santi DV. 1987. Plasmid migration using orthogonal-field-alternation gel electrophoresis. *Nucleic Acids Res.* 15(20):8387-8398.
- Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6(13):3901-3907.
- Johnson FH, Shimomura O, Saiga Y, Gershman LC, Reynolds GT, Waters JR. 1962. Quantum efficiency of *Cypridina* luminescence, with a note on that of *Aequorea*. *J Cell Compar Physl.* 60:85-103.

- Joliot A, Pernelle C, Deagostini-Bazin H, Prochiantz A. 1991. Attennapedia homeobox peptide regulates neural morphogenesis. *P Natl Acad Sci USA*. 88(5):1864-1868.
- Jones AT, Sayers EJ. 2012. Cell entry of cell penetrating peptides: tales of tails wagging dogs. *J Control Release*. 161(2):582-591.
- Kaplan IM, Wadia JS, Dowdy SF. 2005. Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release*. 102:247-253.
- Khalafalla MM, Rahman SM, El-Shemy HA, Nakamoto Y, Wakasa K, Ishimoto M. 2005. Optimization of particle bombardment conditions by monitoring of transient sGFP(S65T) expression in transformed soybean. *Breeding Sci*. 55(3):257-263.
- Klein TM, Wolf ED, Wu R, Sanford JC. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature*. 327:70-73.
- Köhler RH, Zipfel WR, Webb WW, Hanson MR. 1997. The green fluorescent protein as a marker to visualize plant mitochondria *in vivo*. *Plant J*. 11(3):613-621.
- Koornneef M, Meinke D. 2010. The development of *Arabidopsis* as a model plant. *Plant J*. 61(6):909-921.
- Koren E, Torchilin VP. 2012. Cell-penetrating peptides: breaking through to the other side. *Trends Mol Med*. 18(7):385-393.
- Krens FA, Molendijk L, Wullems GJ, Schilperoort RA. 1982. *In vitro* transformation of plant protoplasts with Ti-plasmid DNA. *Nature*. 296:72-74.
- Langel Ü, Pooga M, Kairane C, Zilmer M, Bartfai T. 1996. A galanin-mastoparan chimeric peptide activates the Na⁺, K⁺-ATPase and reverses its inhibition by ouabain. *Regul. Peptides*. 62:47-52.
- Li BJ, Langridge WHR, Szalay AA. 1985. Somatic embryogenesis and plantlet regeneration in the soybean *Glycine max*. *Plant Cell Rep*. 4(6):344-347.
- Lindgren M, Hällbrink M, Prochiantz A, Langel Ü. 2000. Cell-penetrating peptides. *Trends Pharmacol Sci*. 21(3):99-103.
- Lindgren M, Rosenthal-Aizman K, Saar K, Eiríksdóttir E, Jiang Y, Sassian M, Östlund P, Hällbrink M, Langel Ü. 2006. Overcoming methotrexate resistance in breast cancer tumour cells by the use of a new cell-penetrating peptide. *Biochem Pharmacol*. 71(4):416-425.
- Lloyd AM, Barnason AR, Rogers SG, Byrne MC, Fraley RT, Horsch RB. 1986. Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. *Science*. 234(4775):464-466.
- Lörz H, Baker B, Schell J. 1985. Gene transfer to cereal cells mediated by protoplast transformation. *Mol Genet Genomics*. 199(2):178-182.
- Lu DP, Wu SJ, Gao XQ, Zhang YL, Shan LB, He P. 2010. A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *P Natl Acad Sci USA*. 107:496-501.
- Lundberg M, Wikström S, Johansson M. 2003. Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther*. 8:143-150.

- Mäe M, Myrberg H, Jiang Y, Paves H, Valkna A, Langel Ü. 2005. Internalisation of cell-penetrating peptides into tobacco protoplasts. *Biochim Biophys Acta.* 1669(2):101-107.
- Maiolo JR, Ferrer M, Ottinger EA. 2005. Effects of cargo molecules on the cellular uptake of arginine-rich cell-penetrating peptides. *Biochim Biophys Acta.* 1712(2):161-172.
- Meurer CA, Dinkins RD, Redmond CT, McAllister KP, Tucker DT, Walker DR, Parrott WA, Trick HN, Essig JS, Frantz HM, et al. 2001. Embryogenic response of multiple soybean [*Glycine max* (L.) Merr.] cultivars across three locations. *In Vitro Cell Dev-Pl.* 37:62-67.
- Mitchell DJ, Kim DT, Steinman K, Fathman CG, Rothbard JB. 2000. Polyarginine enters cells more efficiently than other polycationic homopolymers. *J Pept Res.* 56(5):318-325.
- Obel N, Erben V, Schwarz T, Kühnel S, Fodor A, Pauly M. 2009. Microanalysis of plant cell wall polysaccharides. *Mol Plant.* 2(5):922-932.
- Palm-Apergi C, Lorents A, Padari K, Pooga M, Hällbrink M. 2009. The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. *FASEB J.* 23:214-223.
- Parry M, Rosenzweig C, Livermore M. 2005. Climate change, global food supply and risk of hunger. *Philos T R Soc B.* 360(1463):2125-2138.
- Pooga M, Hällbrink M, Zorko M, Langel Ü. 1998. Cell penetration by transportan. *FASEB J.* 12:67-77.
- Qi X, Droste T, Kao CC. 2011. Cell-penetrating peptides derived from viral capsid proteins. *Mol Plant Microbe In.* 24:25-36.
- Reissmann S. 2014. Cell penetration: scope and limitations by the application of cell-penetrating peptides. *J Pept Sci.* 20(10):760-784.
- Richmond TA, Somerville CR. 2001. Integrative approaches to determining Csl function. *Plant Mol Biol.* 47:131-143.
- Roest S, Gilissen LJW. 1989. Plant regeneration from protoplasts: a literature review. *Acta Bot Neerl.* 38:1-23.
- Santarem ER, Pelissier B, Finer JJ. 1997. Effect of explant orientation, pH, solidifying agent and wounding on initiation of soybean somatic embryos. *In Vitro Cell Dev-Pl.* 33:13-19.
- Sawant RM, Hurley JP, Salmaso S, Kale A, Tolcheva E, Levchenko TS, Torchilin VP. 2006. "SMART" drug delivery systems: double-targeted pH-responsive pharmaceutical nanocarriers. *Bioconjugate Chem.* 17(4):943-949.
- Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, et al. 2010. Genome sequence of the palaeopolyploid soybean. *Nature.* 463:178-183.

- Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DW. 1995. Green-fluorescent protein as a new vital marker in plant cells. *Plant J.* 8(5):777-784.
- Slater A, Scott N, Fowler M. 2008. *Plant biotechnology*. New York (NY): Oxford University Press.
- Smith EF, Townsend CO. 1907. A plant-tumor of bacterial origin. *Science.* 25(643):671-673.
- Tomlin ES, Branch SR, Chamberlain D, Gabe H, Wright MS, Stewart Jr. CN. 2002. Screening of soybean, *Glycine max* (L.) Merrill, lines for somatic embryo induction and maturation capability from immature cotyledons. *In Vitro Cell Dev-Pl.* 38(6):543-548.
- Trabulo S, Cardoso AL, Mano M, Pedrosso de Lima MC. 2010. Cell-penetrating peptides – mechanisms of cellular uptake and generation of delivery systems. *Pharmaceuticals.* 3(4):961-993.
- [USDA-FAS] United States Department of Agriculture – Foreign Agricultural Service. 2015. Oilseeds: world markets and trade. World Production, Markets, and Trade Reports. [2015 Nov 9; accessed 2015 Nov 22]. <http://www.fas.usda.gov/data/oilseeds-world-markets-and-trade>.
- Vivès E, Brodin P, Lebleu B. 1997. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem.* 272:16010-16017.
- von Arnold A, Sabala I, Bozhkov P, Dyachok J, Filonova L. 2002. Developmental pathways of somatic embryogenesis. *Plant Cell Tiss Org.* 69(3):233-249.
- Wachter RM, Elsliger MA, Kallio K, Hanson GT, Remington SJ. 1998. Structural basis of spectral shifts in the yellow-emission variants of green fluorescent protein. *Structure.* 6(10):1267-1277.
- Wadia JS, Stan RV, Dowdy SF. 2004. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med.* 10:310-315.
- Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. 2000. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *P Natl Acad Sci USA.* 97(24):13003-13008.
- Yamada T, Takagi K, Ishimoto M. 2012. Recent advances in soybean transformation and their application of molecular breeding and genomic analysis. *Breeding Sci.* 61(5):480-494.
- Yang Y, Yang Y, Xie X, Cai X, Zhang H, Gong W, Wang Z, Mei X. 2014. PEGylated liposomes with NGR ligand and heat-activable cell-penetrating peptide-doxorubicin conjugate for tumor-specific therapy. *Biomaterials.* 35(14):4368-4381.
- Yoo SD, Cho YH, Sheen J. 2007. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc.* 2:1565-1572.

- Zhang F, Chen C, Ge H, Liu J, Luo Y, Liu K, Chen L, Xu K, Zhang Y, Tan G, Li C. 2014. Efficient soybean regeneration and *Agrobacterium*-mediated transformation using a whole cotyledonary node as an explant. *Biotechnol Appl Bioc.* 61(5):620-625.
- Ziemienowicz A, Shim YS, Matsuoka A, Eudes F, Kovalchuk I. 2012. A novel method of transgene delivery into triticales plants using the *Agrobacterium* transferred DNA-derived nano-complex. *Plant Physiol.* 158(4):1503-1513.
- Zonin E, Moscatiello R, Miuzzo M, Cavallarin N, Di Paolo ML, Sandonà D, Marin O, Brini M, Negro A, Navazio L. 2011. TAT-mediated aequorin transduction: an alternative approach for effective calcium measurements in plant cells. *Plant Cell Physiol.* 52(12):2225-2235.

Appendices

Supplementary Table 1: FN Lite macro salts and MS micro salts for FN Lite liquid media. Components and respective amounts needed for 1 L of media.

Component	Amount
Ammonium nitrate	800 mg
Potassium nitrate	3.033 g
Ethylenediaminetetraacetic acid (EDTA), disodium dihydrate	37.26 mg
Ferrous sulfate, heptahydrate	27.8 mg
Manganese sulfate	16.9 mg
Potassium phosphate, monobasic, anhydrous	170 mg
Calcium chloride, anhydrous	332.2 mg
Magnesium sulfate, anhydrous	180.7 mg
Boric acid	6.2 mg
Cobalt chloride, hexahydrate	0.025 mg
Cupric sulfate, pentahydrate	0.025 mg
Molybdic acid sodium salt, dihydrate	0.25 mg
Potassium iodide	0.83 mg
Zinc sulfate, heptahydrate	8.6 mg

Supplementary Table 2: Summary of CPP transformation experiment parameters in soybean somatic embryo clusters. Experiment details for trials carried out on soybean somatic embryo clusters using CPP-mediated transformation (n=20 soybean somatic embryo clusters evaluated in each experiment).

Experiment #	Tat₂ Amount (μg)	Plasmid Amount (μg)	Ratio	Incubation Time (hr)	Temperature (°C)
1	20	4	5:1	1	24
2	20	4	5:1	1	24
3	20	4	5:1	1	37
4	20	4	5:1	1	37
5	20	5	4:1	1	24
6	20	5	4:1	1	24
7	20	5	4:1	1	24
8	12	4	3:1	1	24
9	16	4	4:1	1	24
10	20	4	5:1	1	24
11	24	4	6:1	1	24
12	4	1	4:1	1	24
13	12	3	4:1	1	24
14	20	5	4:1	1	24
15	20	5	4:1	1	33
16	20	5	4:1	1	24
17	20	5	4:1	2	24
18	20	5	4:1	3	24
19	20	5	4:1	4	24
20	20	5	4:1	1	24

Curriculum Vitae

Name: Atiyah Sumaiyah Ferouz

Post-secondary Education and Degrees: The University of Western Ontario
London, Ontario, Canada
2008-2013 B.Sc.

The University of Western Ontario
London, Ontario, Canada
2013-Present M.Sc.

Related Work Experience Teaching Assistant
The University of Western Ontario
2013-2015

Poster Presentations:

Lu, M., Ferouz, A., Eudes, F., Way, D.A., Tian, L. (2013) Transient expression of β -glucuronidase in soybean somatic embryos using cell penetrating peptide transfection. Canadian Society of Plant Biologists, Eastern Regional Meeting, University of Toronto Mississauga, Mississauga, ON.

Ferouz, A., Eudes, F., Way, D.A., Lu, M., Tian, L. (2014) The study of cell-penetrating peptide transfection in soybean somatic embryos. Canadian Association of Plant Biotechnology, Annual Meeting, McGill University, Montreal, QC.

Ferouz, A., Eudes, F., Way, D., Tian, L. (2014) An assessment of cell-penetrating peptide transfection in soybean somatic embryos. Canadian Society of Plant Biologists, Eastern Regional Meeting, Guelph University, Guelph, ON.