EVALUATION OF FUSION PEPTIDES AS NOVEL GENE CARRIERS INTO *NICOTIANA BENTHAMIANA* AND *ARABIDOPSIS THALIANA* LEAVES

By

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LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
AID	Arginine-rich Intracellular Delivery
BCA	Bicinchoninic acid
bp	base pairs
$CaCl_2$	Calcium Chloride
CLSM	Confocal Laser Scanning Microscopy
cm	centimeter
CPP	Cell Penetrating Peptide
DLS	Dynamic Light Scattering
EDTA	Ethylenediaminetetracetic acid
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Linear double-stranded Deoxyribonucleic Acid
dsRNA	double-stranded Ribonucleic Acid
g	grams
g/L	gram / litre
g/mol	gram / mol
GFP	Green Fluorescent Protein
GUS	β-Glucuronidase
HCl	Hydrochloric acid
HD	Hydrodynamic Diameter
HEPES	4-(2-hydroxyethyl-1-piperazineethanesulfonic acid)

HPLC	High Performance Liquid Chromatography
kb	kilo base pairs
KCl	Potassium Chloride
KH_2PO_4	Potassium di-Hydrogen Phospate
KOH	Potassium Hydroxide
L	Liter
LB	Luria Bertani medium
M	Molar
MES	2-(N-morpholino) ethane sulfonic acid
mg	milligram
µg	microgram
mL	milliliter
mM	millimolar
µL	microliter
µM	micromolar
µm	micrometer
NaOH	Sodium Hydroxide
nm	nanometer
nmol	nanomol
N/P	Nitrogen / Phosphate ratio
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P35S	Cauliflower Mosaic Virus 35S promoter
pDNA	plasmid Deoxyribonucleic Acid
PDI	Polydispersity Index

RLuc	*Renilla* luciferase
RLU	Relative Light Units
RNA	Ribonucleic Acid
rpm	revolutions per minute
siRNA	short interfering Ribonucleic Acid
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite repression
TAE	Tris-Acetate-EDTA buffer
TNOS	Nopaline Synthase Terminator
UV-Vis	Ultraviolet-Visible Spectroscopy
v/v	volume/volume
w/v	weight/volume

PENILAIAN PEPTIDA GABUNGAN SEBAGAI PEMBAWA GEN YANG BARU KE DALAM DAUN *NICOTIANA BENTHAMIANA* DAN *ARABIDOPSIS THALIANA*

ABSTRAK

Penghantaran gen menggunakan peptida melibatkan proses pengenalan gen asing ke dalam sel haiwan dan tumbuhan melalui peptida berfungsi yang mengandungi domain penembus sel dan jujukan polikation. Peptida penembus sel berkebolehan untuk memasuki sel-sel hidup, sementara jujukan polikation berkebolehan untuk bercantum dan memadatkan plasmid DNA secara elektrostatik. Dalam kajian ini, potensi peptida gabungan yang melingkungi unjuran polikation dan domain berfungsi dengan berkebolehan untuk menembusi sel hidup telah dibentuk dan diuji sebagai pengangkut gen ke dalam sel tumbuhan. Peptida-peptida gabungan yang mempunyai unjuran polikation, nona-arginin (R9) dan ko-polimer histidin-lisina $(KH)_9$ berserta peptida penembus sel Bp100 (KKLFKKILKYL) dan Tat_2 (RKKRRQRRRRKKRRQRRR) telah dicampurkan dengan plasmid DNA (pDNA) yang mengekod *Renilla luciferase* (RLuc) untuk membentuk kompleks pDNA-peptida pada pelbagai nisbah N/P. Nisbah, N/P merujuk kepada nisbah bilangan kumpulan amina daripada peptide per bilangan kumpulan fosfat daripada pDNA. Kompleks tersebut dicirikan dari segi saiz, cas permukaan (potensi zeta), stabiliti and morfologi. Kompleks-kompleks yang telah dihasilkan pada pelbagai nisbah N/P (0.1, 0.5, 1, 2, 5, 10 and 20) dalam air ternyahion telah disusupkan pada permukaan bawah daun *Nicotiana benthamiana* dan *Arabidopsis thaliana* yang berusia 3 minggu dan dikuantifikasikan untuk mengekspres gen RLuc meggunakan '*Renilla luciferase*

assay' pada selang masa yang tertentu sehingga 144 jam. Kompleks yang disediakan pada nisbah N/P 0.5 menunjukkan bentuk globul dengan diameter hidrodinamik dalam lingkungan 300-400 nm dan caj permukaan negatif. Kompleks ini juga telah mencatatkan aktiviti RLuc yang tertinggi pada 12 jam selepas penyusupan bagi semua peptida gabungan $(KH)_9$-Bp100 yang diuji. Berdasarkan pemerhatian yang diperoleh daripada kajian peringkat pertama ini, kompleks pDNA-peptida telah diuji dengan lebih lanjut meggunakan pelbagai jenis larutan penampan dengan nilai pH yang berbeza. Untuk kajian ini, kompleks-kompleks secara terlebih dahulu dibentuk pada pelbagai nisbah N/P (0.1, 0.5, 1, 2 and 5) dalam larutan penampan dan dicirikan dari segi saiz, caj permukaan dan morfologi. Kajian efisiensi transfeksi telah dilakukan ke atas daun *N. benthamiana* dan keputusan kajian ini telah mencadangkan bahawa kompleks pDNA-peptida pada nisbah N/P 0.5 masih menunjukkan efisiensi yang terbaik dalam larutan penampan (30 mM PBS, pH 7.4) dan menunjukkan aktiviti yang setanding dengan keputusan yang diperoleh dalam air ternyahion. Dalam peringkat ketiga, DNA bebenang ganda dua (dsDNA) yang mengekod RLuc telah disintesis menggunakan teknik PCR dengan menggunakan pDNA RLuc sebagai templat. DNA ini telah digunakan untuk membentuk kompleks dsDNA-peptida dalam air ternyahion dan larutan penampan yang terdiri daripada pelbagai nisbah N/P. Kompleks dsDNA-peptida pada nisbah N/P 1 memberikan efiensi transfeksi yang tertinggi dalam kedua-dua kondisi air ternyahion dan larutan penampan (30 mM PBS, pH 7.4).

EVALUATION OF FUSION PEPTIDES AS NOVEL GENE CARRIERS INTO *NICOTIANA BENTHAMIANA* AND *ARABIDOPSIS THALIANA* LEAVES

ABSTRACT

Peptide-based gene delivery involves introduction of foreign genes into animal and plant cells via functional peptides, containing cell penetrating domains and polycationic sequences. Cell penetrating domains are capable of being internalized into living cells, while polycationic sequences can electrostatically interact and condense DNA. In this study, three fusion peptides consisting of polycationic sequences and functional domains with cell penetrating ability were designed and evaluated as potential gene carriers for plant cells. The fusion peptides, consisting of nona-arginine (R9) and histidine-lysine $(KH)_9$ polycationic sequences as well as Bp100 (KKLFKKILKYL) and Tat_2 (RKKRRQRRRRKKRRQRRR) cell penetrating domains respectively were mixed with pDNA encoding *Renilla* luciferase (RLuc) to form pDNA-peptide complexes at various N/P ratios. Here, N/P ratio refers to the ratio of number of amines from peptides per number of phosphates from pDNA. The complexes were characterized in terms of size, surface charges (zeta potential), stability and morphology. The complexes prepared at various N/P ratios (0.1, 0.5, 1, 2, 5, 10 and 20) in deionized water were infiltrated at the abaxial section of 3-week old *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves and quantified for RLuc gene expression using RLuc assay at various time points up to 144 hours. Complexes of $(KH)_9$-Bp100 prepared at N/P ratio 0.5 demonstrated globular shapes with hydrodynamic diameters between 300-400 nm and negatively charged surface. This

complexes also showed the highest transfection efficiencies at 12 hours after infiltration for all the fusion peptides compared to the other N/P ratios. Based on the observation obtained in this first section, the pDNA-peptide complexes were further evaluated in different buffers and pH. For this, complexes were first prepared at various N/P ratios (0.1, 0.5, 1, 2 and 5) and characterized in terms of size, surface charge and morphology. Transfection efficiency studies performed on *N. benthamiana* leaves suggested that pDNA-peptide complexes at N/P ratio 0.5 still showed the highest levels of efficiencies in buffer solution (30 mM, PBS, pH 7.4) along with the results obtained in deionized water. In the third stage, a linear double stranded DNA (dsDNA) encoding RLuc genes were synthesized using PCR methods with the pDNA encoding RLuc as the template and used to prepare dsDNA-peptide complexes in both deionized water and buffers at various N/P ratios. The dsDNA-peptide complexes at N/P ratio 1 gave highest transfection efficiencies in both deionized water and buffer conditions (30 mM, PBS, pH 7.4).

CHAPTER 1

1.0 INTRODUCTION

In the world of plant science research, plant genetic transformation is a common process, as it is routinely executed in many labs either to understand the plant behaviour or harness its huge genetic potentials. What started off as a successful routine experiment by Estrella-Hererra (1983) has since come a long way into the current state, where genetically modified plants have become part of a billion dollar industry in this world. According to a report by Nature in 2013, as of 2012 a total number of 170 million hectares of genetically modified crops have been planted worldwide and the number has been growing ever since. The global value of genetically modified seed was at a staggering 15 billion USD in 2012. These statistical reports have clearly shown, that growing genetically modified crops are becoming a common but increasingly important practice globally. With the largely expanding human population and rapidly changing climate around the globe, the fundamental need to produce high quality and tolerant crops to sustain the human population in this world has arisen. One most effective way to achieve high quality crops is by genetically engineering the plants in order to improve/modify its native traits to become tolerant against harsh environmental conditions and/or produce high yield of products. Plant transformation has been used to improve the native crop traits in order to become pest, disease and herbicide resistant (Toenniessen et al., 2003, Funke et al., 2006, Vaughn et al., 2005) as well as to modify the crops to become draught tolerant (Hu and Xiong, 2014). Besides that, nutritional content in food based crops were significantly improved using genetic engineering techniques (Paine et al., 2005, Newell-McGloughlin, 2008).

Achieving a successful genetically transformed plant requires reliable and reproducible methods in genetic engineering. A good transformation method will always ensure that plants can be routinely transformed at high efficiencies. Numerous methods have been applied to genetically transform plants, but out of these large options, there are two methods widely used to consistently transform plants. These methods have been superior over other available techniques in achieving successful genetically transformed plant. They are *Agrobacterium*-mediated transformation and particle bombardment or gene gun method (Newell, 2000). *Agrobacterium*-mediated transformation employs the plant pathogen *Agrobacterium tumefaciens* to transfer genes into plants using its T-DNA harbouring the genes of interest (Ziemienowicz, 2014). Many successful plant transformations were achieved using this method due to its ability to transfer large intact DNA, simple transgene insertions and stable integration and inheritance. However, this technique remains largely recalcitrant for many monocot plants due to its species limitations (Barampuram and Zhang, 2011). Particle bombardment or gene gun method uses DNA coated high velocity microprojectiles to deliver genes into plants. This technique has countered the species specificity issue raised in *Agrobacterium* mediated transformation, as many important monocot plants such as wheat, corn and rice were successfully transformed using this method. Besides that, this method has enabled organelle transformation in plant cells such as chloroplast, mitochondria and nucleus (Kikkert et al., 2005). Despite resolving the species limitation issue and initiating organelle targeting, this method has several drawbacks, due to its transformation efficiency compared to *Agrobacterium*-mediated transformation, high cost of equipment, tendency for complex integration and multiple copy insertions and gene and tissue damage due to high velocity or vacuum intolerance (Kikkert et

al., 2005). The common requirements for transformation systems for practical plant genetic engineering are ready availability of the target tissue, applicability to a wide range of plant types, high efficiency in terms of economy and reproducibility, technical simplicity such that it does not require demanding procedures or equipment and safety to operators as in no dangerous or hazardous operation procedures (Birch, 1997). It is therefore essential to kick start a new method which could encompass all these requirements and become a potentially leading next generation strategy for an enhanced plant genetic transformation.

Peptide-based gene delivery method has been extensively studied and showed tremendous success in animal cell system (Simeoni et al., 2003, Numata and Kaplan, 2010a). Whilst having a huge potential, this technique however is poorly studied in plant cells with only a few reports citing success in the delivery of plasmid DNA (pDNA) using cell penetrating peptides (CPP) into permeabilized immature wheat embryo (Chugh and Eudes, 2008), mung bean and soy bean roots (Chen et al., 2007) and double stranded RNA (dsRNA) into tobacco suspension cells to induce post-transcriptional gene silencing (Unnamalai et al., 2004). Furthermore, the permeability and transfection activities via the peptide-based delivery has not been properly quantified in the studies mentioned above. The peptide-based gene delivery system could be advantageous by having no species limitation issue and can potentially be applicable in any type of plants. Besides that, the delivery of genes via this system does not require any costly equipment or special plant preparations such as protoplasting and has low risk of gene damage since no strong mechanical forces are involved in aiding the gene delivery.

It was previously found in animal cells that fusion peptides consisting of silk protein and CPP are effective as gene carriers both *in vivo* and *in vitro* (Numata and

Kaplan, 2010b, Numata et al., 2011b). Furthermore, the transfection efficiencies was found to be greatly enhanced when functional peptide such as tumour homing domain was added into the fusion peptides. This showed that the presence of functional peptides on the surface of the pDNA-peptide complexes helped to increase the transfection efficiencies (Numata et al., 2011a). Therefore in this study, fusion peptides with DNA binding polycationic sequences along with CPP were designed. The polycationic sequences or cationic homopeptides will preferentially interact electrostatically with DNA molecules to form condense complexes due to its high cationic charge density, while the CPP, which has lower extent of DNA binding capability than cationic homopeptides will aid the cellular entry of the complexes (Figure 1). These fusion peptides were tested for their ability to deliver genes *in vivo* into intact plant cells. They were evaluated on the leaves of two model plants, *Nicotiana benthamiana* and *Arabidopsis thaliana* in deionized water platform. The outcomes from this first stage of study were used to choose the peptides and model plants to be used in the subsequent stages. In the second and third stages, the peptide-based gene carrier was evaluated in buffer conditions at various pH and the ability of the peptide to carry genes in various DNA forms (plasmid DNA and linear double-stranded DNA).

Therefore, the main objectives of this study are:

a. To design fusion-peptides capable of binding DNA into complexes and deliver into plant cells
b. To determine the physicochemical properties of the plasmid DNA (pDNA)-peptide and linear double stranded DNA (dsDNA)-peptide complexes in deionized water and buffer platforms.

c. To test (quantitative and qualitative) the ability of the fusion-peptide to deliver genes into *N. benthamiana* and *A. thaliana* leaves.

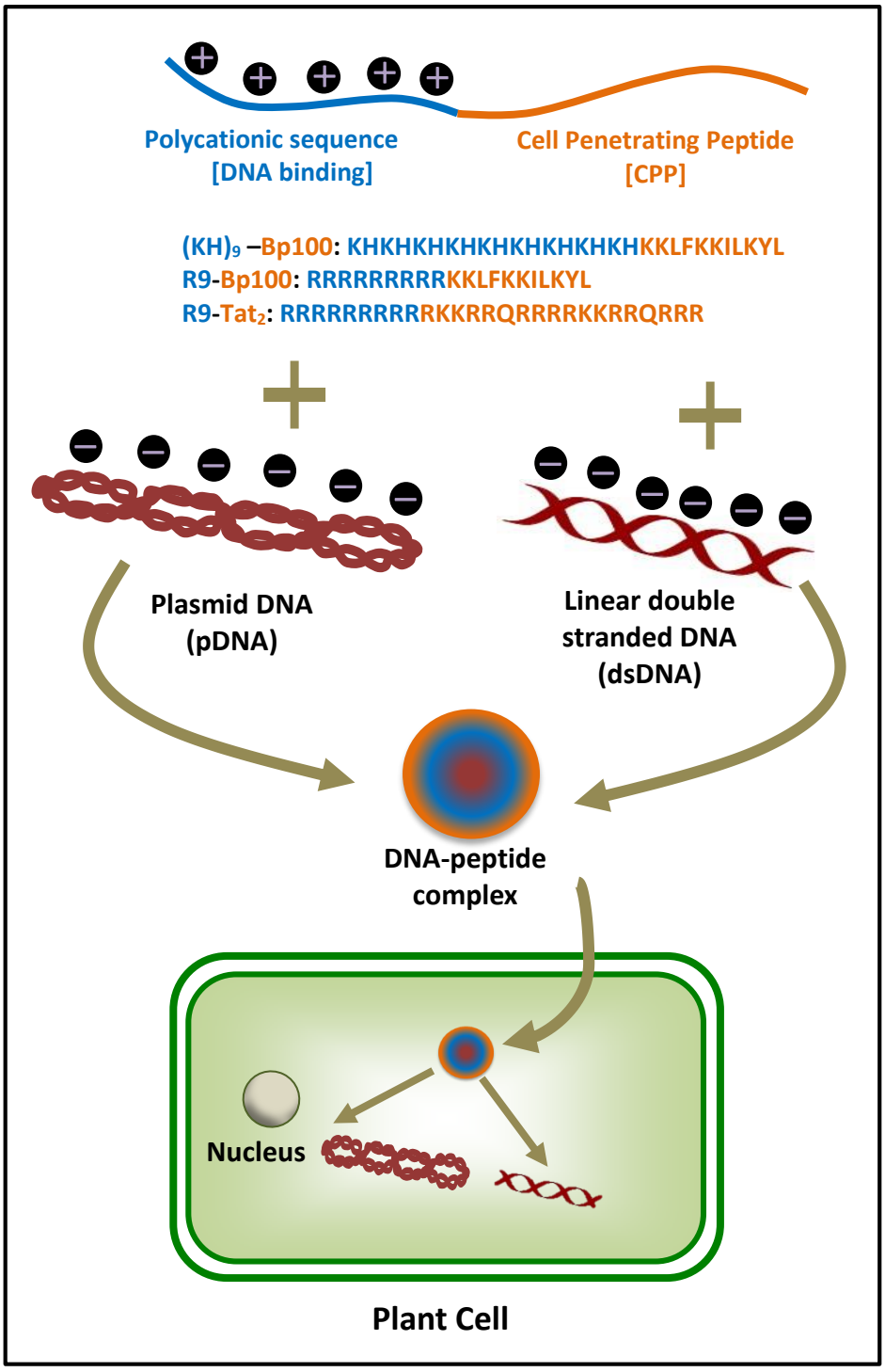

Figure 1: The peptide-based gene delivery system. (a) Fusion peptides $(KH)_9$-Bp100, R9-Bp100 and R9-Tat_2 used in this study and negatively charged pDNA and dsDNA. (b) Formation of DNA-peptide complexes when pDNA or dsDNA is mixed with fusion peptide via electrostatic interactions. (c) Upon infiltration into leaves, the complexes with the aid of CPP internalizes into the plant cells and the DNA is released and expressed throughout the cell. Figure is not drawn to scale.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Common plant genetic transformation methods

Plant transformation in general refers to the process of introduction of foreign genes into plant cells, which in later stages are taken up by the host genome via homologous recombination and stably expressed by the plant or transiently expressed without homologous recombination (Newell, 2000). Either way, both transformations are equally important in various areas of plant research. Various methods have now become available to perform plant genetic transformations. However, this chapter will emphasize on the two most commonly used methods to achieve successful plant transformations. These methods are *Agrobacterium*-mediated transformation and biolistics or particle bombardment method (Barampuram and Zhang, 2011, Rivera et al., 2012).

2.1.1 *Agrobacterium* mediated transformation

Originally discovered as a plant pathogen, *Agrobacterium tumifaciens* is a Gram-negative soil bacterium, which has a capability to infect and induce crown gall tumours at the wounded regions of dicotyledonous plants (Kado, 1991, Zambryski, 1992, Hooykaas and Beijersbergen, 1994). The process of tumour induction occurs when *Agrobacterium* transfers part of its 200-800 kb Ti plasmid, the T-DNA into plant cells. The T-DNA is flanked by a 24 bp T-DNA border sequences which are highly homologous (Gerard et al., 1992, Fortin et al., 1993, Goodner et al., 2001). The map of the typical Ti plasmid and the T-DNA region is shown in Figure 2.1. The

process of T-DNA transfer is regulated by a series of virulence (*vir*) genes, which are induced by compounds secreted by wounded plant cells, such as acetosyringone (AS) (Winnans, 1992). T-DNA along with several vir proteins are exported into the plant cells via the VirB/D4 type IV secretion system (Christie, 2004). The *vir* region possesses eight operons, namely *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, *virG*, and *virH* encoding proteins to regulate the transfer of T-DNA into host cells. For genetic transformation purposes, the genes of interest is placed between left and right border repeats of T-DNA (Gelvin, 2003) and the T-DNA harbouring the genes of interest is stably transformed into the host cell using following mechanisms (a) microhomology-based integration of single-stranded T-DNA or (b) integration of double-stranded T-DNA into double strand breaks (Ziemienowicz, 2014).

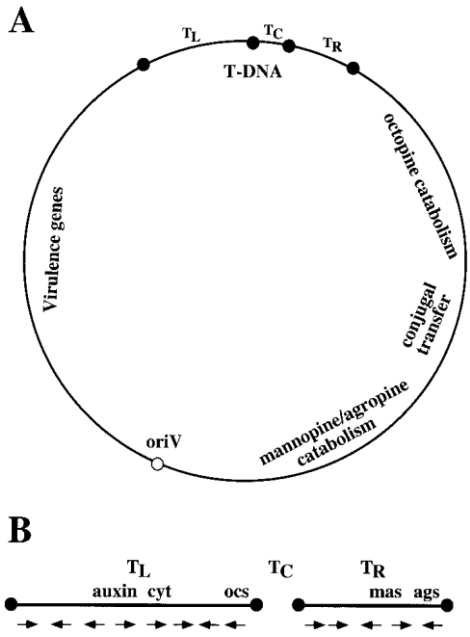

Figure 2.1: Schematic representation of a typical octopine-type Ti plasmid (A) and the T-DNA region of a typical octopine-type Ti plasmid (B). (A) The T-DNA is divided into three regions. TL (T-DNA left), TC (T-DNA center), and TR (T-DNA right). The black circles indicate T-DNA border repeat sequences. *oriV*, the vegetative origin of replication of the Ti plasmid, is indicated by a white circle. (B) The various T-DNA-encoded transcripts, and their direction of transcription, are indicated by arrows. Genes encoding functions involved in auxin synthesis (auxin), cytokinin synthesis (cyt), and the synthesis of the opines octopine (ocs), mannopine (mas), and agropine (ags) are indicated (Gelvin, 2003).

Plant proteins are known to contribute significantly to *Agrobacterium*-mediated transformation. BTI1, VIP1, Ku80, CAK2Ms, histones H2A,H3-11 and H4, SGA1, UDP glucosyltransferase, and GALL S interacting proteins were reported to be involved in T-DNA and virulence protein transfer, cytoplasm trafficking, nuclear targeting, T-DNA integration, stability and expression, and defense responses (Ziemienowicz, 2014). The transformation efficiency using *Agrobacterium* method is governed by several factors, which includes genotype of the plant, plasmid vector, bacterial strain, composition of culture medium, tissue damage, suppression and elimination of *Agrobacterium* infection after co-cultivation (Kavitah et al., 2010, Sood et al., 2011). The summary of these factors have been listed in detail in review by Ziemienowicz (2014) and is shown in Table 2.1.

Agrobacterium-mediated transformation was first used on model plants such as *Arabidopsis thaliana*, *Medicago trunculata*, *Nicotiana tabacum* and *Nicotiana benthamiana*. Over the years, the plant range which showed successful transformation using *Agrobacterium* has expanded largely. This includes some monocot plants which were previously recalcitrant to this method. The categories of plants in this list include cereal crops, legumes, industrial crops, vegetables, turf grass, woody plants, root plants, tropical plants, nuts and fruits, ornamental plants and medicinal plants (Figure 2.2).

Table 2.1: Factors influencing *Agrobacterium*-mediated plant transformation (Ziemienowicz, 2014)

Factors	Examples
Explant type	Root, shoot, cotyledon, embryo, hypocotyl
Vector plasmid	pCAMBIA, pGreen, pGA, pCG, pGPTV, Bi-BAC, etc.
Bacterial strain	LBA4404, EHA101 ,C58, AGL1
Composition of culture medium	Salt concentration, sugars, growth regulators
Temperature of co-cultivation	Range: 19–30 °C; optimal temp. dicots:19–20 °C, monocots: 24–25 °C
Time of co-cultivation	1–5 days; common: 24h, 48h, 60h, 72h
Agrobacterium density	1×10^{6}–1×10^{10} cfu/ml
pH of co-cultivation medium	Acidic pH: 5.2, 5.5, 5.6, 5.8 or 6.0
Antibiotics	Cefotaxime, carbenecillin, kanamycin, timentin
Chemicals	Acetosyringe, L-cysteine, dithiothreitol and sodium thiosulphate
Surfactants	Silwet L77, pluronic acid F68, Tween20
Selectable markers	*hpt*, *pat*, *nptII*[a]

[a] *hpt* hygromycin phosphotransferase gene, *pat* phosphinothricin acetyl transferase gene, *nptII* neomycin phosphotransferasegene

Model plants	Arabidopsis (*Arabidopsis thaliana*), barrel clover (*Medicago truncatula*), tobacco (*Nicotiana benthamiana*, *N. tabacum*
Cereal crops	Barley (*Hordeum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), rye (*Secale cereal*), sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*)
Legume	Alfalfa (*Medicago sativa*), chickpea (*Cicer arietinum*), clovers (*Trifolium* spp.), peas (*Pisum sativum*), peanut (*Arachis hypogaea*), pigeon pea (*Cajanus cajan*), soybean (*Glycine max*), beans (*Phaseolus* spp.)
Industrial crops	Canola (*Brassica napus*), Cotton (*Gassypium hirsutum*), Indian mustard (*Brassica juncea*), sunflower (*Helianthus annus*)
Vegetable plants	Cabbage (*Brassica oleracea*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), lettuce (*Letuca sativa*), tomato (*Lycopersicum esculentum*)
Root plants	Carrot (*Daucus carota*), cassava (*Manihot esclenta*), potato (*Solanum tuberosum*), sweet potato (*Ipomoea batatas*)
Turf grasses	Bermuda grass (*Cynodon* spp.), perennial ryegrass (*Lolium perenne*), switch grass (*Panicum virgatum*), tall fescue (*Festuca arundinacea*), bent grass (*Argostis* spp.)
Tropic plants	Banana (*Musa* spp.), *Citrus* spp., coffee (*Coffea* spp.), papaya (*Carica papaya*), pineapple (*Ananas comosus*), sugarcane (*Saccharum* spp.)
Woody species	American elm (*Ulmus americana*), cork oak (*Quercus suber*), *Eucalyptus*, pine (*Pinus radiate*), poplar (*Populus* spp.), rubber trees (*Hevea brasiliensis*)
Nuts and fruits	American chestnut (*Castanea dentata*), apple (*Malus* x *domestica*), blueberry (*Vaccinium corymbosum*), grapevine (*Vitis vinifera*), strawberry (*Fragaria* x *ananassa*)
Ornamental plants	Carnation (*Dianthus caryophylus*), chrysanthemum (*Dendrathema* x *glandiflora*), orchids (*cymbidium* spp., *Oncidium*, *Phalaenopsis*), petunia (*Petunia hybrida*), rose (*Rosa hybrida*)
Medicinal plants	Ginseng (*Panax ginseng*), hemp (*Cannabis sativa*), opium poppy (*Papaver somniferum*)

Figure 2.2: Categories and examples of plant species transformed by *Agrobacterium* (Ziemienowicz, 2014)

2.1.2 Microprojectile/Particle bombardment method

The particle bombardment method employs high metal particles or known as projectiles to deliver intact DNA into plant cells. This method was first introduced by Sanford in 1987 (Sanford, 1987). It was later, when Klein and co-workers identified the huge potential of this method. They found that tungsten microparticles could be used to coat nucleic acids such as DNA/RNA and delivered and transiently expressed in onion epidermal cells (Klein et al., 1987). Following that, Christou and co-workers (1988) reported stable transformation of soy bean callus using particle bombardment method. From this novel discovery, the term biolistics (biological ballistics) was officially adapted for the process and device introduced by Sanford. Biolistic® is a registered trademark of E.I du Pont Nemours and Co., which is now sold under the auspices of Bio Rad Laboratories, Hercules, CA. The most widely used particle bombardment device is the Biolistic ® PDS-1000/He particle delivery system marketed by Bio Rad Laboratories. This system utilizes high pressure helium released by a rupture disk to mobilize a macrocarrier sheet loaded with millions of DNA coated gold/tungsten microparticles toward target cells (Kikkert et al., 2005). A stopping screen holds the macrocarrier and the microcarrier continue to fly towards the target cells. The graphical summary of the Biolistic® PDS-1000/He particle is shown in Figure 2.3 and the bombardment mechanism is shown in Figure 2.4.

The particle bombardment method is applicable to wide variety of tissues and intact cells due to its simple methodology. In plants, this method has been used to achieve transient gene expressions, production of genetically transformed plant and inoculation of plants with viral pathogens. Besides that, organellar gene delivery was established using this method.

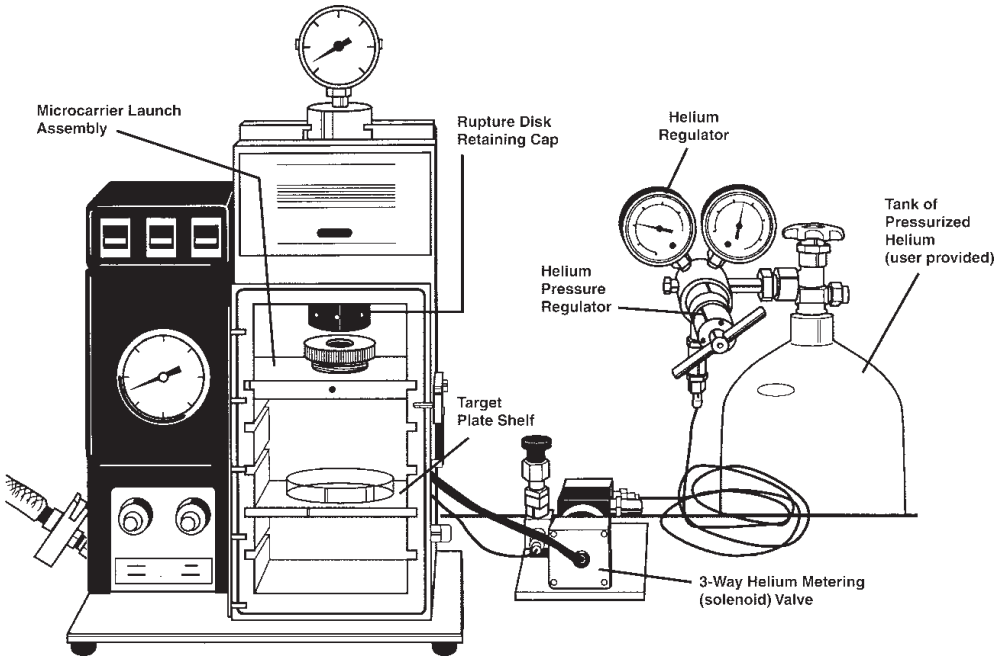

Figure 2.3: Components of the Biolistic® PDS-1000/He particle delivery system. (Drawing courtesy of Bio-Rad Laboratories, Hercules, CA.)(Kikkert et al., 2005)

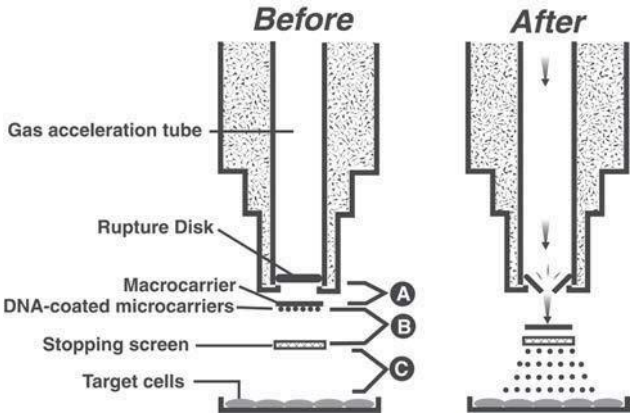

Figure 2.4: Biolistic® PDS-1000/He bombardment process. The velocity of the macrocarriers is dependent on the helium pressure in the gas acceleration tube, the distance from the rupture disk to the macrocarrier (gap distance) (A), the macrocarrier travel distance to the stopping screen (B), the distance between the stopping screen and target cells (C), and the amount of vacuum in the bombardment chamber. (Drawing courtesy of Bio-Rad Laboratories, Hercules, CA.)(Kikkert et al., 2005)

Organelles such as chloroplasts, mitochondria and nucleus of important monocot crops (wheat, corn and rice) were successfully transformed using this technique. As with any plant transformation method, several parameters need to be optimized for the process to be maximally effective. With particle bombardment, the parameters can be grouped as physical, biological, and environmental (Southgate et al., 1995, Taylor and Fauquet, 2002) Physical parameters include the composition and size of the microcarriers, the attachment of DNA to the microcarriers, and several instrument parameters. The first biological parameter to consider is a gene construct in the form of a circular or linear plasmid or a linear expression cassette (promoter–gene–terminator). It is important to match the promoter or other regulatory sequences with the plant tissue, so that the gene will be expressed at desired levels. Other biological parameters include tissue type, cell size, cell culture age and general cellular health, target tolerance of vacuum, cell density, and cell turgor pressure. The physiological status of the target influences receptivity to foreign DNA delivery and susceptibility to injury that may adversely affect the outcome of the transformation process (Kikkert et al., 2005).

Environmental factors such as temperature; humidity; and light intensity, quality, and duration have a direct effect on tissue physiology and thus transformation success (McCabe and Christou, 1993). In addition, some explants may require a “healing” period after bombardment under special regimens of light, temperature, and humidity (McCabe and Christou, 1993). Humidity also is important in microcarrier preparation and bombardment. High humidity can cause the microcarriers to clump and/or to bind irreversibly to the macrocarrier, thus reducing transformation rates. High humidity may also affect alcohol stocks used during the DNA/microcarrier coating steps.

The physical nature of the biolistic process eliminates concerns about using another biological organism in the transformation process. In grapevines, there is often a hypersensitive response to *Agrobacterium* that causes plant cell death (Perl et al., 1996). Particle bombardment method removes both the need to kill *Agrobacterium* after transformation and the occurrence of false positives arising from growth of *Agrobacterium* in the host tissues. Furthermore, plasmid construction is often simplified and co-transformation with multiple transgenes (Francois et al., 2002) is routine, because plasmid DNA is simply mixed together before coating onto the microcarriers. The use of linear expression cassettes (also called clean gene technology) eliminates the chance that extraneous plasmid backbone DNA will be inserted into the target as can happen with whole plasmids or *Agrobacterium* (Fu et al., 2000). Particle bombardment is the method of choice for the study of transient gene expression and for plastid transformation (Taylor and Fauquet, 2002). Some disadvantages of this method are that the transformation efficiency may be lower than with *Agrobacterium* and the device and consumables are costly. Many researchers did not want to consider particle bombardment because of the tendency for complex integration patterns and multiple copy insertions that could cause gene silencing. Some researchers have overcome this problem by reducing the quantity of DNA loaded onto the microcarriers or by use of linear cassettes (Fu et al., 2000). Table 2.2 shows some examples of important crops that have been successfully transformed using particle bombardment method

Table 2.2: Selected transgenic plants produced by biolistics reviewed by (Rivera et al., 2012)

Crop	Crop
Maize	Sugar cane
Tobacco	Barley
Rice	Cow pea
Carrot	Peanut
Petunia	Chickpeas
Sorghum	Alfalfa
Brassica napus	Spruce
Potato	Conifer
Wheat	Pine
Grass	Eucalyptus
Tomato	Fescue
Sugar Beet	Soybean
Legume	*Arabidopsis*
Cotton	Strawberry
Algae	Cereals
Banana	Papaya
Onion	Garlic
Bean	Nut
Grape	Cassava
Triticale	Oat
Millets	Chrysanthemum
Rose	Orchid
Jute	Linum
Rape	Rye
Lesquerella	Betalain
Lettuce	Lemon fruit tree
Citrus	Palm
Silver birch	Coffee
Pepper	Moss
Powlownia	

2.2 Peptide-based gene delivery: A fast-expanding method in plant cell research

Cell penetrating peptides (CPP) is a special class of peptide which is short (30 amino acids long), cationic or amphipathic in nature, contains predominantly lysine and arginine amino acids, easy to prepare and generally non-cytotoxic. They have the innate ability to reach cytoplasmic/nuclear regions in live cells after internalization (Joliot and Prochiantz, 2004). The first CPP discovered in 1988 was derived from the Transactivator of Transcription (Tat) of Human Immunodeficiency Virus 1 (HIV-1) (Green and Loewenstein, 1988, Frankel and Pabo, 1988). This peptide has 11 amino acid sequences (YGRKKRRQRRR), which is responsible for the cellular entry of Tat (Vives et al., 1997). In the later years, more CPPs were derived from natural (protein), synthetic and chimeric sources. Peptide-based gene delivery system stood out from the other polymeric gene delivery methods due to their innate ability to effectively overcome the cell's lipid bilayer membrane barrier, while carrying a cargo along. Together with that, they have been found to be sufficiently stable for *in-vivo* applications (Amantana et al., 2007, Weiss et al., 2007, Neundorf et al., 2008, Pujals et al., 2008) and are more potent with higher specificity and low toxicology concerns (Lindgren and Langel, 2011).

Plant cells differ from animal cells in two major ways. One is their photosynthetic capability and the second is the presence of a cell wall surrounding the cell membrane. The latter has a complex structure made of simple sugar polymers, cellulose and hemicellulose, and lignin. Although rigid, its nature changes with the cell cycle to facilitate growth and division. Younger cells tend to have a thinner cell wall than mature cells (Chugh et al., 2010). The first published evidence that peptides specifically may be used for macromolecular delivery into plant cells was reflected in the studies which showed that core histone proteins had cellular

penetration attributes in petunia protoplasts and suspension cultured cells (Rosenbluh et al., 2004). Core histone proteins (e.g., H2A, H2B, H3 and H4) share the primary characteristics of CPPs with high cationic properties and DNA binding affinity, suggesting that CPPs may also be applied to plant cells. Despite the potential advantages of CPPs, plant cells have some morphological and chemical differences to that of mammalian cells which can render a portion of the knowledge obtained from mammalian cell studies of little benefit. These differences include, but are not limited to, membrane lipid composition (fatty acids, head groups, etc.), the cellulose cell wall, rates of endocytosis, membrane signalling systems and media conditions for tissue culture. Even with these several potentially confounding factors, common CPPs that have been successful in mammalian cell systems, like Tat, have also shown success in plant tissue culture systems. However, this success has been limited and there is a growing demand to establish an understanding of how CPPs interact specifically with plant cells and how to improve their efficacy (Ziemienowicz et al., 2015).

Much like how it has been found in other non-mammalian cell types, CPPs in the absence of a bound cargo are often capable of energy independent translocation across the cell membrane of plant cells. In a study comparing Bowes human melanoma cells to tobacco SR-1 protoplasts it was found that Transportan displayed the highest translocation efficiency compared to *p*VEC, Penetratin and TP10 in the tobacco protoplasts. Despite a clear indication of translocation this efficiency was much lower than translocation of these same CPPs in the cultured melanoma cells (Mae et al., 2007). The reasons for this large difference in efficiency are not yet clear, but are likely related to the chemical make-up of the plasma membrane of the protoplasts. Further work in protoplasts was seen in triticale mesophyll protoplasts

using Tat and Tat_2, a proprietary dimer of Tat (Chugh and Eudes, 2007). Not only was translocation observed but accumulation in the nucleus was also found in both Tat and Tat_2, with Tat_2 displaying 1.6 times higher build up. Interestingly, lowering of temperature of incubation to 4 °C was found to increase accumulation by nearly double what was found at 25 °C, indicating an inclination towards an energy independent mechanism of uptake. Although penetration was much less than that in mammalian cell systems, it is interesting to note that CPP uptake has been observed in both monocot and dicot protoplasts with similar results (Chugh and Eudes, 2007, Chugh and Eudes, 2008a). However, delivery in protoplasts does not address a systemic layer of complexity intrinsic to plant cells, the plant cell wall. It must be recognized that the plant cell wall presents a unique challenge to CPP translocation due to its ubiquity in practical tissue culture systems (e.g., induced callus, cell suspension and microspores) and, of course, in mature plants. The plant cell wall consists primarily of polysaccharides, the principle one being cellulose in somatic tissue systems. The cell wall presents both a physical and chemical barrier to the use of CPP in plant cells. It acts as an adsorptive surface to highly cationic CPPs likely due to a slightly negative charge (Mizuno et al., 2009).

This adsorptive behavior can reduce the concentration of the solubilized CPPs and thereby reduce the amount that can make contact with the membrane and therefore translocate. It has also been shown that permeabilization of the cell wall increases uptake of CPPs in immature wheat embryos (Chugh and Eudes, 2008b) indicating that the diameter of the cell wall pores and permeability of the cellulose matrix are, not surprisingly, factors in efficient uptake. Furthermore, CPPs derived from *Brome mosaic virus* (BMV) capsid protein (CPNT, CP9-22, and CP12-22) showed significant translocation and accumulation within cells of *Arabidopsis* and

barley root hairs, but nearly ubiquitous accumulation at the cell wall, further reinforcing the issue of the cell wall (Qi et al., 2011). In non-somatic tissue, particularly in isolated cereal microspores, the translocation of free peptide has also been verified. In the case of triticale microspores, translocation of free peptide was observed using fluoresceinated Tat, Tat_2 , *p*VEC, and Transportan (Chugh et al., 2009). It was shown that Transportan translocated in the greatest amounts despite having little cationic character compared to the other peptides tested. This data seems to suggest that significant basic or cationic character alone is not sufficient for translocation of peptides across the plant cell membrane. Microspores themselves present a valuable model platform for these studies as they possess an immature exine, which is (by comparison to the cellulosic cell wall of somatic tissues) very impermeable, except for the micropore, a region of exposed membrane (Chugh et al., 2009). Additionally, the exine is composed of a chemically disparate polymer called sporopollenin, which is far more flexible and has more negative charge character than cellulose (Kim and Douglas, 2013, Lallemand et al., 2013). These characteristics exacerbate the already present concerns of the cellulosic cell wall. Despite these heightening, translocation was still demonstrated in measurable quantities, strongly indicating the potential of CPPs in this tissue culture system as well. In order to increase the efficiency of CPP translocation, the plant cell wall remains a primary issue of interest.

It has been established that protein above 25 kDa is unable to cross the cell wall, preventing macromolecules from interacting with the cell membrane and entering the cell through endocytosis (Stewart et al., 2008). In soybean cell suspension cultures, the major constraint limiting macromolecule endocytosis was also the size of the molecule (Horn et al., 1992). A negative slope log-linear

relationship was found between the molecular weight of the macromolecule and its rate of internalization, with no uptake above 150 kDa (IgG). Recent investigations showed that peptides, such as Tat, Tat_2, arginine-rich intracellular delivery peptides (AID), pVEC, transportan, and penetratin, can be taken up by suspensions of protoplasts from tobacco cells and triticale mesophyll cells (Chugh and Eudes, 2007, Chugh and Eudes, 2008a, Unnamalai et al., 2004, Chen et al., 2007). The uptake of pVEC and transportan in various plants tissues were also studied (Chugh and Eudes, 2008a). Among the various plant tissues studied, pVEC and transportan showed significantly weaker fluorescence in leaf tips than coleoptiles excised from 7-day-old triticale seedlings. However, leaf bases that are highly meristematic in nature and the root tips known for high mitotic activity showed significant internalization of both pVEC and transportan as observed by fluorescence microscopy.

Covalent and noncovalent transduction of small 24 kDa fluorescent reporter proteins by Tat-PTD and arginine-rich intracellular delivery (AID) proteins in mung bean, soybean, corn, and onion root tip cells were reported by Chang and coworkers (Chang et al., 2005, Chang et al., 2007). The cell wall remains a major obstacle for transduction of larger proteins in plant tissues, such as immature scutellum and cotyledon, a model system for genetic transformation studies owing to their amenability toward tissue culture procedures and high efficacy for plant regeneration (Chugh et al., 2010). Permeabilization of cell wall is a prerequisite to achieve efficient translocation of CPPs and their macromolecular cargoes (Chugh and Eudes, 2008b). Following such pretreatment, non-labeled Tat monomer (Tat) and dimmer (Tat_2) are able to deliver the large protein β-glucuronidase (GUS, Mw 270 kDa) efficiently. The permeabilization treatment of the immature embryos also increased the intensity of the blue color. Commercially available Chariot kit (pep-1 as the

carrier peptide) for protein delivery in mammalian cell lines delivered GUS enzyme in wheat embryos efficiently. Chariot kit has also been used for direct delivery of bacterial avirulence (Avr) proteins into resistant *Arabidopsis* protoplasts resulting in hypersensitive cell death in a gene for gene-specific manner (Wu et al., 2003). Low temperature (4 °C) treatment of the permeabilized embryos resulted in low GUS enzyme activity, indicating that endocytosis is involved in Tat_2-mediated cargo translocation, as temperatures below 10 °C are known to inhibit endocytic pathways in cells (Chugh and Eudes, 2008b). This was validated using inhibitors of endocytosis and macropinocytosis. Both type of inhibitors caused reduction in GUS enzyme activity; however, no conclusive picture emerged that could enable us to determine involvement of a specific pathway in the uptake of cargo complex in immature embryos (Chugh and Eudes, 2008b). More than one mechanism appear to be involved simultaneously for the uptake of noncovalent cargo complex in somatic plant cells and may involve both endocytosis and macropinocytosis pathways. However, micropinocytosis was the mechanism of uptake of fluorescent protein covalently linked to AID peptide (Chang et al., 2005). Tat_2, AID, and R12 being arginine rich can bind DNA electrostatically resulting in complex formation that can be used for gene delivery in the plant cells.

Successful nucleic acid (DNA and dsRNA) transfection has been reported in protoplast, tobacco cell suspension culture, mungbean and soyabean root tips, and permeabilized immature wheat embryos (Unnamalai et al., 2004, Chugh and Eudes, 2007, Chen et al., 2007, Chugh and Eudes, 2008b). Posttranscriptional gene silencing was achieved using sense and antisense constructs of 0.4 and 0.9 kb in dsRNA/R12-treated tobacco cell suspension culture (Unnamalai et al., 2004). The authors detected a 21-bp small interfering RNA. Transient GUS gene expression in

permeabilized immature embryos showed that Tat_2 can efficiently cross cell wall and translocate plasmid DNA in somatic plant cells amenable to regeneration of plantlets. The complex size at the optimal ratio (4:1) of Tat_2 and plasmid DNA varied between 0.85 and 4 μm (Chugh and Eudes, 2008b).

2.3 Genesis of nucleic acid-peptide complexes

In the peptide-based gene delivery system, the key factor which determines the success of gene delivery in living cells is the interaction or attachment of cargo to be delivered with the peptide. The peptide has to pack the cargo into condensed and compact shapes before they could be brought across the cell wall and membrane barrier. This interaction can be achieved either covalently or non-covalently. There are various covalent methods used to bind the cargoes to peptides, such as sulfosuccinimidyl suberate linkage, carbodiimide conjugation, and thiol-amine coupling (Huang et al., 2015). Covalent methods were mostly used to conjugate drugs, antibody fragments and fluorescent markers. Although a stable association between the cargo and peptide could be achieved using this technique, the covalent-linking procedure is however very time consuming, expensive and labour intensive. Furthermore, in order to achieve covalent linking, the functionality of the peptide-cargo complex could be compromised (Huang et al., 2015). The first report on the successful delivery of peptide-fusion protein into various tissue sections in mice via covalent linking was from Schwarze and co-workers in 1999 (Schwarze et al., 1999). Subsequently, several reports were published on the successful delivery of nucleic acids into various cell lines via covalent method (Sebestyen et al., 1998, Snyder and Dowdy, 2004, Nan et al., 2005, Ciolina et al., 1999).

Non-covalent interactions on the other hand were achieved using biotin-streptavidin interactions, electrostatic interactions, and metal-affinity interactions. The advantages of using non-covalent techniques are, ease of use, simplicity in production, versatility in terms of cargo composition and retention of cargo functionality (Chang et al., 2014, Huang et al., 2015). Using this methods, nucleic acids, oligonucleotides and other biomolecules such as collagen, insulin and fluorescent proteins (Wang et al., 2006, Hou et al., 2007, Meade and Dowdy, 2007, Eguchi and Dowdy, 2009, Chen et al., 2012). Figure 2.5 shows the pictorial summary of both covalent and non-covalent methods in the binding between peptide and target cargoes. This thesis will focus primarily on the binding of peptide with DNA using electrostatic interactions and a more comprehensive review on the binding mechanism will be covered in this chapter.

Electrostatic interaction by definition is the bond that is achieved when a cationic molecule comes together with an anionic molecule via attraction of opposite charges. When charges from both molecules are cancelled off, a stable product or complex is formed. This same concept is applied in the binding of nucleic acids with peptides using electrostatic interactions. A nucleic acid molecule, be it a DNA, RNA or siRNA are negatively charged due to their phosphate backbone. A peptide, particularly CPPs and domains with polycationic peptides such as lysine, histidine and arginine are positively charged due to the amine group from the peptides. When nucleic acids are deliberately mixed with cationic peptides, both molecules will electrostatically interact, causing the nucleic acid molecules to be folded and condensed into compact rods or globular/toroidal shapes by the peptides. (Hansma et al., 1998, Vijayanathan et al., 2004, Osada et al., 2010, Osada et al., 2012). Understanding the folding mechanism of the nucleic acid has been a great interest

lately due to the ongoing efforts to enhance gene delivery and expression in living cells. In the reports published by Osada and group in 2010 and 2012 respectively, a simple mechanism on the condensation process of catiomers on pDNA have been proposed. The quantized folding model as termed by them showed that the supercoiled double helix of pDNA collapses into rod structure and undergoes further folding in a specific manner.