DEVELOPMENT OF A PEPTIDE-BASED SYSTEM FOR DELIVERY OF Cas9:sgRNA COMPLEXES INTO *Arabidopsis thaliana*

NG KIAW KIAW

UNIVERSITI SAINS MALAYSIA

2017

DEVELOPMENT OF A PEPTIDE-BASED SYSTEM FOR DELIVERY OF Cas9:sgRNA COMPLEXES INTO *Arabidopsis thaliana*

by

NG KIAW KIAW

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

December 2017

ACKNOWLEDGEMENT

First of all, I would like to express my utmost thanks and gratefulness to my USM supervisors, Prof. Dr. Ahmad Sofiman bin Othman and Prof. Dr. K. Sudesh Kumar for giving me the great opportunity to conduct my PhD research in RIKEN Wako Campus, Japan. They are extremely helpful and supportive throughout my PhD study. They have provided me with valuable advices, patience and tireless effort in order to help me succeed through my professional and personal difficulties.

Secondly, I would also like to express my deepest gratitude to my RIKEN supervisor, Dr. Keiji Numata for his supervision and guidance throughout the period of my PhD research conducted in Enzyme Research Team, RIKEN Center for Sustainable Resource Science. He has provided me a very well-equipped laboratory for my PhD study and financially supported me to participate in several international conferences. I am very grateful that he has imparted me with many invaluable knowledge and experience in my PhD project and scientific writing.

Besides, I would like to extend my multifold thanks and sincerest gratitude to Dr. Yutaka Kodama and Ms. Yoko Motoda for their kind assistance in the plasmid construction and cell-free protein synthesis, respectively. Meanwhile, my special thanks to Dr. Chuah Jo-Ann, Dr. Lin Ting-Yu and Dr. Ali Malay for their valuable comments and suggestions in improving my research and scientific writing skills. In addition, I would also like to express my sincere gratitude to Dr. Chayatip Insomphun, Dr. Takeshi Yoshizumi, Ms. Yoko Horii, Wong Yoke Ming, Dr. Nicholas Thomson and Dr. Jose Manuel Ageitos who never fail to offer me friendly assistance in research.

Not to forget the other members of Enzyme Research Team (Dr. Kosuke Tsuchiya, Dr Mieko Higuchi, Dr. Kenjiro Yazawa, Dr. Nur Alia Oktaviani, Ms. Ayaka Tateishi, Ms. Nao Ifuku, Madam Kumiko Morisaki and Madam Hiroe Watanabe) for their kind support and caring.

I would like to extend my thankfulness to secretaries of Enzyme Research Team (Madam Akiko Irie and Madam Yuki Tsujimoto), secretaries of Synthetic Genomics Research Group (Ms. Nobuko Kimura and Ms. Mariko Ikeda) and the staffs of RIKEN Global Relations Office (Mr. Motohide Yokota, Ms. Chiharu Shimoyamada, Ms. Maya Kobayashi, Ms. Shinko Saito, Ms. Yoko Fujita, Ms. Ai Suzuki and Ms. Mihoko Morihata) for their great effort and support in international students' affairs and living.

Apart from that, I would like to dedicate my thankfulness to my friends in USM (Ng Lee Mei, Mok Pei Shze, Wong Kok Tong and Siti Nurfazilah) for their generous assistance in many ways. Moreover, I would like to thanks to all the staffs of School of Biological Sciences, USM, especially Madam Sarifah binti Hanafie for their kind assistance throughout my PhD study.

I would also like to acknowledges MyPhD scholarship and RIKEN International Program Associate Fellowship for the financial supports. This research project was funded by Cross-ministerial Strategic Innovation Promotion Program (SIP), Japan.

Last but not least, I would like to express my special gratitude to my parents, brother and fiancé for their unconditional love, moral support and encouragement since the commencement of my PhD study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
MES	2-(N-morpholino)ethanesulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DAPI	4',6-diamidino-2-phenylindole
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
GUS	β-glucuronidase
A_{280}	Absorbance at 280 nm
A_{555}	Absorbance at 555 nm
ADH	Alcohol dehydrogenases
N-	Amine-
ANOVA	Analysis of variance
AMPs	Antimicrobial peptides
ca.	Approximately
Arg	Arginine
AID	Arginine-rich intracellular delivery
AFM	Atomic Force Microscopy
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
C-	Carboxyl-
CPP	Cell penetrating peptide
cm	Centimeter
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CLSM	Confocal Laser Scanning Microscopy
CBPs	Consecutive base pairs
Cas	CRISPR-associated
CPT	Covalent protein transduction
Da	Dalton
d	Day

^{o}C	Degree Celcius
DOL	Degree of labelling
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DMSO	Dimethyl sulfoxide
EDTA	Disodium Ethylene Diamine Tetra-Acetate
DTT	Dithiothreitol
DSB	Double-strand break
DLS	Dynamic Light Scattering
EtBr	Ethidium bromide
EDTA	Ethylenediamine tetra-acetic acid
e.g.	exempli gratia
F	Forward
GAG	Glycosaminoglycan
g	Gram
g/L	Gram/litre
g/mol	Gram/mole
GFP	Green fluorescent protein
H	Histidine
He-Ne	Helium-Neon
HDR	Homology directed repair
HSD	Honest significant difference
h	Hour
HCl	Hydrochloric acid
IBPs	Internal base pairs
IBM	International Business Machines Corporation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
pI	Isolelectric point
kbp	Kilobase pair
kDa	Kilodalton
Leu	Leucine
LB	Lysogeny broth

K	Lysine
Lys	Lysine
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
$MnCl_2$	Manganese (II) chloride
Mpa	Megapascal
mRNA	Messenger RNA
ng	Nanogram
µg	Microgram
µL	Microlitre
µM	Micrometre
mA	milliampere
mg	Milligram
mL	Millilitre
mM	Millimolar
mV	millivolt
min	Minute
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
MW	Molecular weight
MAP	Model amphipathic peptide
MS	Murashige and Skoog
nm	Nanometer
nM	Nanomolar
NIH	National Institute of Health
N/m	Newton/meter
DMF	N.N-dimethylformamide
CNPT	Non-covalent protein transduction
NHEJ	Non-homologous end-joining
N/A	Not applicable
nDNA	Nuclear deoxyribonucleic acid

NLS	Nuclear localization signal
nt	Nucleotide
No	Number
n	Number of replicates
OD	Optical density
PTS	Peroxisomal targeting signal
PBS	Phosphate-buffered saline
PIPES	Piperazine-1,4-bis[2-ethanesulfonic acid]
pDNA	Plasmid DNA
PDI	Polydispersity Index
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
KCl	Potassium chloride
KOH	Potasium hydroxide
Pro	Proline
pVEC	Peptide vascular endothelial-cadherin
RhoA	Ras homolog family A
Rac1	Ras-related C3 botulinum toxin substrate 1
RFP	Red fluorescent protein
R	Reverse
rpm	Revolution per minute
RITC	Rhodamine-B-isothiocyanate
RNase	Ribonuclease enzyme
RNA	Ribonucleic acid
RbcL	Ribulose-1,5-bisphosphate carboxylase large subunit
PAGE	Polyacrylamide gel electrophoresis
PAM	Protospacer adjacent motif
PDS	Phytoene desaturase
Sec	Second
Ser	Serine
SKL	Serine-Lysine-Leucine
sgRNA	Single guide RNA

NaCl	Sodium chloride
SDS	Sodium dodecyl sulfate
S.D.	Standard deviation
SPSS	Statistical Package for the Social Sciences
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite Repression
SAP	Sweet arrow peptide
TBPs	Total base pairs
TALENs	Transcription activator-like effector nucleases
Tpl	Tachyplesin
UV	Ultraviolet
U	Unit
Val	Valine
Ver.	Version
V	Volt
H_2O	Water
YFP	Yellow fluorescent protein
ZFNs	Zinc-finger nucleases

LIST OF SYMBOLS

α	Alpha
°	Degree
<	Less than
ε1%	Mass extinction coefficient
ε	Molar extinction coefficient
%	Percentage

PERKEMBANGAN SISTEM BERASASKAN-PEPTIDA BAGI PENGHANTARAN KOMPLEKS Cas9:sgRNA KE DALAM *Arabidopsis thaliana*

ABSTRAK

Pada masa kini, sistem CRISPR/Cas9 telah muncul sebagai alat penyunting genom yang berkuasa untuk pelbagai spesies tumbuhan, disebabkan ciri-cirinya yang mudah dan serba boleh. Biasanya, dalam sistem tumbuhan, Cas9 dan sgRNA dihantar ke dalam sel tumbuhan dalam bentuk DNA. Walau bagaimanapun, penghantaran DNA boleh menjadi rumit atas masalah-masalah seperti penyepaduan DNA asing yang tidak dijangka ke dalam genom tumbuhan, pengoptimuman kodon dan pemilihan promoter untuk pelbagai spesies tumbuhan. Untuk mengatasi masalah ini, sistem penghantaran langsung dari kompleks Cas9:sgRNA diperlukan untuk penyuntingan genom yang disasarkan di dalam tumbuhan. Walaupun terdapat laporan mengenai penghantaran langsung Cas9:sgRNA kompleks dalam sistem protoplast dan embrionik, namun tidak ada laporan mengenai sistem penyampaian kompleks Cas9:sgRNA yang berjaya dijalankan di tumbuhan utuh, disebabkan struktur hierarkinya. Oleh itu, kajian ini bertujuan untuk membangunkan sistem penyampaian berasaskan peptida untuk kompleks Cas9:sgRNA ke dalam sel *Arabidopsis thaliana* utuh untuk penyuntingan genom yang disasarkan. Dua pembawa protein, $(BP100)_2K_8$ dan $BP100(KH)_9$, telah direka berdasarkan gabungan peptida penembusan sel (CPP), iaitu dimer BP100 atau BP100, dan peptida polikationik, iaitu copolymer alternatif lisin dan histidin, $(KH)_9$ atau lapan residu resin berturutan, K_8. Sebagai langkah awal untuk mencapai tujuan

ini, protein bercaj negatif dengan pelbagai berat molekul, termasuk citrine (27 kDa), albumin serum lembu (BSA, 66 kDa) dan alkohol dehidrogenase (ADH, 150 kDa) digunakan sebagai model protein untuk mengoptimumkan keadaan penghantaran berasaskan peptida dan untuk menilai kebolehan sistem penghantaran protein berasaskan peptida. Di samping itu, dua protein berkonjugat dengan peptida yang menyasarkan organel, isyarat lokalisasi sitrin-nuklear (NLS) dan isyarat yang menyasarkan sitrin-peroksisomal (PTS), digunakan untuk menyiasat sama ada peptida gabungan itu mengganggu fungsi peptida yang menyasarkan organel. Hasilnya menunjukkan bahawa protein pembawa yang direka mampu membawa protein dengan pelbagai berat molekul (27 hingga 150 kDa) ke dalam sel-sel *A. thaliana* yang utuh tanpa mengganggu peptida yang menyasarkan organel yang berkonjugat dengan protein. Seterusnya, sistem penyampaian protein berasaskan peptida yang optima telah digunakan untuk penyuntingan genom CRISPR dalam transgenik *A. thaliana* yang mengekspresikan protein pendarfluor kuning (YFP). Kompleks Cas9:sgRNA yang menyasarkan gen YFP dihantar ke sel tumbuhan melalui protein pembawa untuk mendorong mutasi pada gen YFP. Penghantaran kompleks Cas9:sgRNA telah ditunjukkan dapat mendorong mutasi titik di tapak sasaran sgRNA, menyebabkan kehilangan YFP pendarfluor. Kesimpulannya, peptida gabungan yang direka mampu membawa kompleks Cas9:sgRNA ke dalam tumbuhan utuh untuk menjalankan penyuntingan genom yang disasarkan. Sistem penyampaian berasaskan peptida untuk kompleks Cas9:sgRNA boleh digunakan sebagai alat berpotensi tinggi dalam penyuntingan genom tumbuhan.

DEVELOPMENT OF A PEPTIDE-BASED SYSTEM FOR DELIVERY OF Cas9:sgRNA COMPLEXES INTO *Arabidopsis thaliana*

ABSTRACT

Nowadays, CRISPR/Cas9 system has emerged as a powerful genome editing tool in various plant species, due to its simplicity and versatility. Typically, in plant system, Cas9 and sgNA are delivered into the plant cell in the form of DNA. However, the DNA delivery can be complicated with several problems, such as unexpected integration of exogenous DNA into plant genome, codon optimization and selection of promoter in respect to various plant species. To overcome these problems, a direct delivery system of Cas9:sgRNA complexes is needed for targeted genome editing in plants. Although there have been reports of the direct delivery Cas9:sgRNA complexes in protoplast and embryonic systems, there are no reports of the successful Cas9:sgRNA complexes delivery system in intact plant, owing to its hierarchical structures. Hence, the current study is aimed to develop a peptide-based delivery system for pre-assembled Cas9:sgRNA complexes into intact *Arabidopsis thaliana* for targeted genome editing. Two protein carriers (fusion peptides), $(BP100)_2K_8$ and $BP100(KH)_9$, were designed based on the fusion of a cell penetrating peptide (CPP), i.e., BP100 or BP100 dimer, and a polycationic peptide, i.e., alternate copolymer of lysine and histidine, $(KH)_9$ or eight consecutive lysine residues, K_8. As an initial step to achieve this aim, negatively-charged proteins with various molecular weights, including citrine (27 kDa), bovine serum albumin (BSA) (66 kDa) and alcohol dehydrogenase (ADH) (150 kDa) were used as model proteins to optimize the peptide-

based delivery conditions and to evaluate feasibility of peptide-based protein delivery system. In addition, two proteins conjugated to organelle-targeting peptides, Citrine-nuclear localization signal (NLS) and Citrine-peroxisomal targeting signal (PTS), were used to investigate whether the fusion peptide interfered with the function of organelle-targeting peptides. The results demonstrated that the designed fusion peptides were able to deliver proteins with a wide range of molecular weights (27 to 150 kDa) into the cells of intact *A. thaliana* without interfering with the organelle-targeting peptide conjugated to the protein. Next, the optimized peptide-based protein delivery system was applied for CRISPR genome editing in transgenic *A. thaliana* expressing yellow fluorescent protein (YFP). The Cas9-sgRNA complex targeting the YFP gene was delivered into the plant cell via fusion peptide to induce mutation at YFP gene. The delivered Cas9:sgRNA complexes were shown to induce point mutations at sgRNA targeting site, resulting in the loss of YFP fluorescence. In conclusion, the designed fusion peptide is able to deliver the Cas9-sgRNA complex into intact plant to perform the targeted genome editing. This novel peptide-based delivery system for Cas9:sgRNA complexes can be used as a highly potential tool in plant genome editing.

CHAPTER ONE – INTRODUCTION

Nowadays, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system which is a bacterial RNA-directed adaptive immune system (Barrangou et al., 2007; Makarova et al., 2006) has been utilized as a robust genome editing tools in diverse plant species, including *Arabidopsis thaliana*, rice, tobacco, poplar, wheat, sorghum, maize, lettuce, tomato, liverwort, and orange (Fan et al., 2015; Jia and Wang, 2014; Jiang et al., 2013; Sugano et al., 2014; Upadhyay et al., 2013). CRISPR/Cas9 system is rapidly superseding other genome editing technologies, e.g., zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), owing to its simplicity and versatility. In respect of each new target site, CRISPR/Cas9 system can be customized by replacing only the single guide RNA (sgRNA) component (Gasiunas et al., 2012) while ZFNs and TALENs require elaborate design and assembly of individual DNA-binding proteins (Boch et al., 2009; Maeder et al., 2008).

Typically, the introduction of Cas9 and sgRNA into plant cells is achieved by the delivery of DNA encoding Cas9 and sgRNA via particle bombardment (Klein et al., 1987; Sanford et al., 1987), *Agrobacterium*-mediated transformation (Schell and Van Montagu, 1977) or polyethylene glycol (PEG)-mediated protoplast transformation (Negrutiu et al., 1987). As compared to protein delivery, the DNA delivery is commonly used due to the ease of preparation, the stability and the smaller size of plasmid DNA. However, the DNA delivery has several drawbacks, including

the random insertion of exogenous DNA into the plant genome, codon optimization and selection of promoter with respect to various plant species. As an alternative to DNA delivery, sgRNA and messenger ribonucleic acid (mRNA) encoding Cas9 could be delivered intracellularly for transient expression. However, owing to the labile nature of mRNA, the application of mRNA delivery system is very limited. To circumvent these problems, it is crucial to develop a delivery system of pre-assembled Cas9:sgRNA complexes into plant cells for targeted genome editing.

In animal system, the direct delivery of pre-assembled Cas9:sgRNA complexes is extensively developed (Kim et al., 2014; Liang et al., 2015; Lin et al., 2014; Ramakrishna et al., 2014; Sun et al., 2015; Zuris et al., 2015). In contrast, the direct delivery of Cas9:sgRNA complexes in plant is still scarcely developed. To date, there are only three reports on the direct delivery of Cas9:sgRNA complexes into protoplast derived from *A. thaliana*, tobacco, rice, lettuce (Subburaj et al., 2016; Woo et al., 2015) as well as maize embryo cells (Svitashev et al., 2016). To the best of my knowledge, there are currently no methods for the direct delivery of Cas9:sgRNA complexes into intact plants, owing to their hierarchical structure. For a simple, rapid and promising genome editing application, it is necessary to develop a delivery system of Cas9:sgRNA complexes in intact plants as this can avoid the laborious and time-consuming cell culture and plant regeneration. Hence, the current study is aimed to develop a feasible peptide-based system for the delivery of pre-assembled Cas9:sgRNA complexes into intact plant for genome editing. The feasibility of the peptide-based protein delivery system was evaluated in intact *Arabidopsis thaliana*.

In current study, cell penetrating peptide (CPP)-fused to a polycationic peptide, which is termed as fusion peptide, were used as protein delivery carrier. Previous

studies demonstrated that fusion peptides showed higher gene delivery efficiency as compared with that of the CPP alone in intact plant (Lakshmanan et al., 2013; Numata, 2015). By using the fusion peptides, the negatively charged cargo molecules preferentially interact with the polycationic peptide through electrostatic interactions, whereas the CPP interacts with fewer cargo molecules and is preferentially present on the surface of peptide-cargo complexes. The presence of many CPPs at the surface of the complexes leads to a higher efficiency in gene delivery (Numata et al., 2012). It was assumed that polycationic peptides also can interact with negatively-charged protein cargoes via electrostatic interaction, similarly to the electrostatic interaction with plasmid DNA as described in previous studies (Lakshmanan et al., 2013). Hereby, two fusion peptides, $(BP100)_2K_8$ and $BP100(KH)_9$, were designed by fusing a CPP, i.e., BP100 or BP100 dimer, and a polycationic peptide, i.e., alternate copolymer of lysine and histidine, $(KH)_9$ or eight consecutive lysine residues, K_8. The fusion peptide, $BP100(KH)_9$ has been reported to be an efficient nuclei acid carrier in previous study (Lakshmanan et al., 2013). On the other hand, $(BP100)_2K_8$ was designed based on the assumption that second BP100 may act as a linker to prevent the poly-lysine (K_8) from interfering the α-helical conformation of the first BP100, which is a crucial structure for the cellular internalization (Wadhwani et al., 2014).

As an initial step to achieve the aim, bovine serum albumin (BSA, 66 kDa) was selected as a model protein to optimize the conditions for peptide-based delivery into the cytosol, such as type of fusion peptides, peptide/protein molar ratio (the protein molarity remained constant, and the peptide molarity increased from 1 to 25) and time course effect. The feasibility of the optimum protein delivery condition with protein cargoes with higher molecular weight was further demonstrated by the delivery of alcohol dehydrogenase (ADH, 150 kDa) into the cytosol. Subsequently, the

compatibility of the optimum peptide-mediated delivery system with the delivery of proteins to specific cellular organelles was demonstrated by using the fluorescent protein Citrine (27 kDa) conjugated to either a nuclear localization signal (NLS) or a peroxisomal targeting signal (PTS). The negatively charged proteins (BSA, ADH, Citrine, Citrine-NLS and Citrine-PTS) interacted with cationic fusion peptide via electrostatic interaction to form peptide/protein complexes that can be delivered into the cells of intact *A. thaliana* (Figure 1.1).

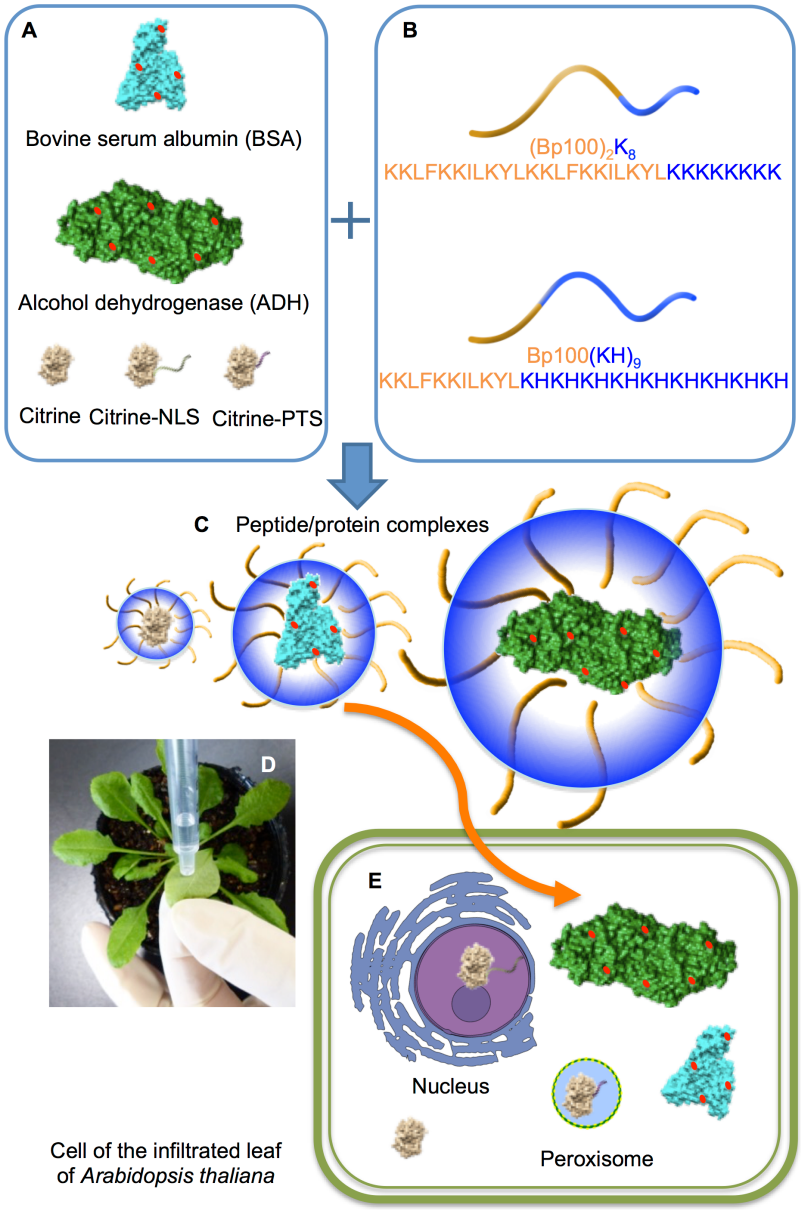

Figure 1.1: Scheme of the fusion-peptide-mediated intracellular delivery of protein cargoes into the leaves of intact *Arabidopsis thaliana*. (A) The protein cargoes were as follows: bovine serum albumin (BSA) (66 kDa), alcohol dehydrogenase (ADH) (150 kDa), Citrine (27 kDa), Citrine with nuclear localization signal (Citrine-NLS) (27 kDa) and Citrine with peroxisomal targeting signal (27 kDa) (Citrine-PTS). BSA and ADH were labelled with Rhodamine B isothiocyanate (red circle). Citrine, Citrine-NLS and Citrine-PTS are fluorescent proteins. (B) The cell-penetrating peptide was BP100, and the polycationic peptides were K_8 or $(KH)_9$. The designed fusion peptides were $(BP100)_2K_8$ and $BP100(KH)_9$. (C) Each protein was mixed with $(BP100)_2K_8$ or $BP100(KH)_9$ at various peptide/protein molar ratios to form the peptide/protein complexes. (D) Infiltration of peptide/protein complexes into *A. thaliana* leaves. (E) The protein complexes penetrated through the cell wall and cell membrane and localized to the cytoplasm or were imported into the peroxisome or nucleus, depending on the fused organelle-targeting peptide.

Next, the applicability of optimum peptide-based protein delivery system for the CRISPR/Cas9 genome editing in transgenic *A. thaliana* that constitutively expressing yellow fluorescent protein (YFP) was investigated. As illustrated in Figure 1.2, the anionic pre-assembled Cas9 Cas9:sgRNA complexes interacted electrostatically with cationic fusion peptide to form $(BP100)_2K_8$/Cas9:sgRNA complexes, and then delivered into the cells of intact YFP *A. thaliana* for *yfp* gene editing via non-homologous end-joining (NHEJ) pathway.

This study represents the first attempt to deliver exogenous proteins and pre-assembled Cas9:sgRNA complexes directly into intact plant via fusion peptides, which represents a novel platform for a safer, more rapid and more promising plant biotechnological applications.

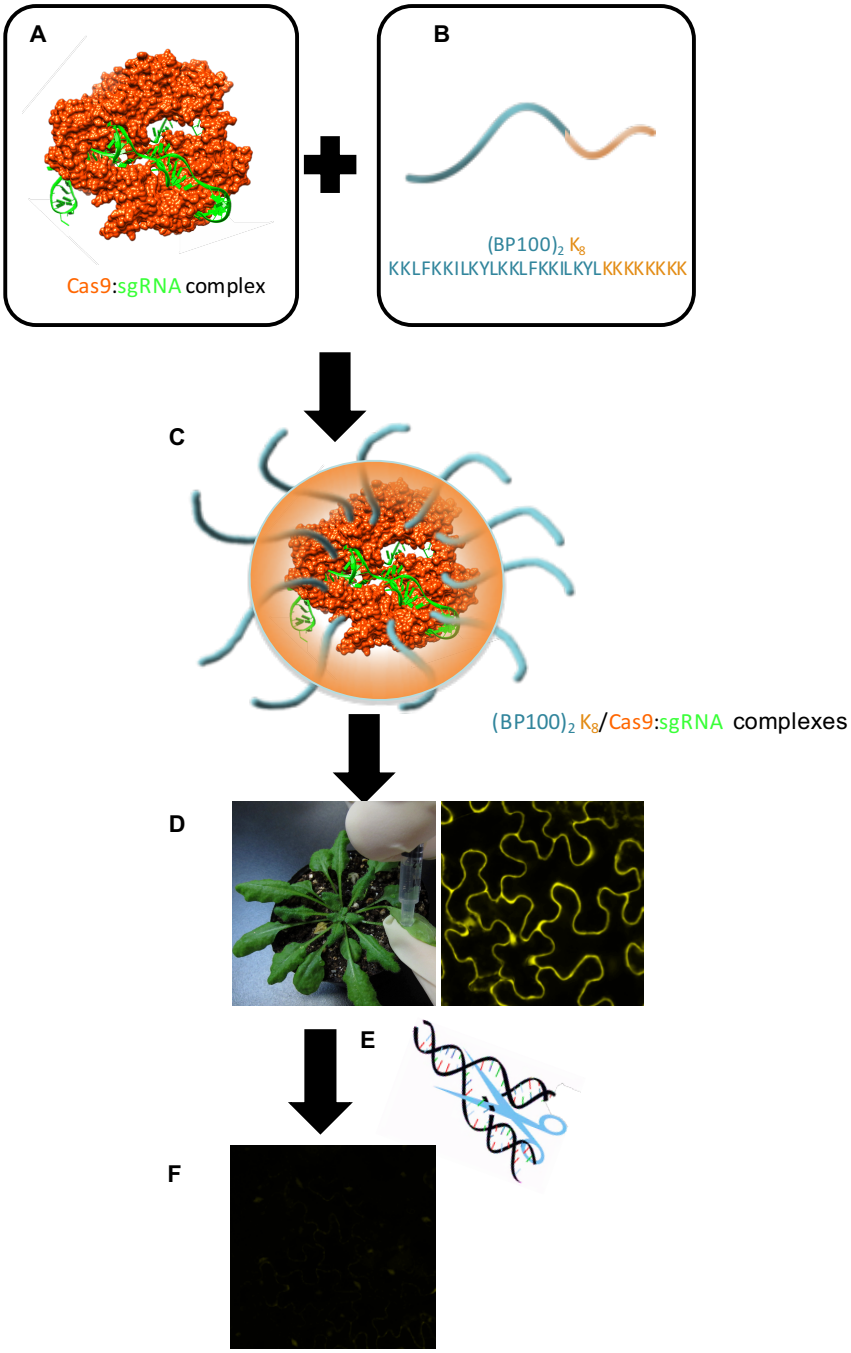

Figure 1.2: Scheme of the fusion-peptide-mediated intracellular delivery of Cas9:sgRNA complexes into the leaves of intact transgenic *Arabidopsis thaliana* that constitutively expressing yellow fluorescent protein (YFP). (A) 100 nM of Cas9 was incubated with twice molar excess of sgRNAs to form Cas9:sgRNA complexes. (B) The cell-penetrating peptide was BP100, and the polycationic peptides were K_8. The designed protein carrier was $(BP100)_2K_8$. (C) The *in vitro* pre-assembled Cas9:sgRNA complexes were mixed with ten molar excess of $(BP100)_2K_8$ to form the $(BP100)_2K_8$/Cas9:sgRNA complexes. (D) Infiltration of $(BP100)_2K_8$/Cas9:sgRNA complexes into leaves of transgenic YFP *A. thaliana*. (E) The delivered Cas9:sgRNA complexes cleaved the target site at *yfp* gene, resulting in the targeted DNA repair by endogenous NHEJ pathway and site-specific mutation. (F) The resulting mutation led to the loss of YFP fluorescence.

CHAPTER TWO - LITERATURE REVIEW

2.1 Common Plant Genetic Transformation Methods

The genetic transformation is a powerful tool for plant agriculture as it allows the introduction of desirable traits into existing plant genome while preserving genetic identity of the plants. Currently, the genetic modification of plants is primarily accomplished by the delivery of exogenous DNA encoding a desired trait. Over the years, a wide variety of methods have been reported for the introduction of exogenous gene into plant genomes. The genetic transformation method can be classified into two groups: (i) indirect gene transfer, which involves the introduction of exogenous DNA in the form of plasmid vector; and (ii) direct gene transfer, which involves the utilization of physical or chemical method for the intracellular delivery of exogenous DNA. The three most commonly used transformation methods are *Agrobacterium*-mediated transformation (indirect gene transfer system) (Schell and Van Montagu, 1977), polyethylene glycol (PEG)-mediated protoplast transformation (direct gene transfer system) (Negrutiu et al., 1987) and particle bombardment (direct gene transfer system) (Klein et al., 1987; Sanford et al., 1987).

2.1.1 *Agrobacterium*-Mediated Transformation

Agrobacterium-mediated transformation has been successfully applied to economically and horticulturally important monocotyledonous and dicotyledonous species. Some examples of the plant species that are successfully transformed by *Agrobacterium tumefaciens* can be found in Ziemienowicz (2014).

A. tumefaciens is a soil phytopathogen which transfers a portion of its DNA, T-DNA (transferred DNA) to the host genome and causes crown gall tumour on the host plant (Schell and Van Montagu, 1977). The genetic components of *A. tumefaciens* that are required for plant cell transformation include the T-DNA that is transported from the bacterium to the plant cell, the Ti plasmid virulence (*vir*) region which is the master switch for transformation, and chromosomal virulence (*chv*) genes which are responsible for the attachment of bacteria to the wounded plant cell (Stachel et al., 1985; Stachel et al., 1986). The T-DNA region is located on the 200-kb of Ti plasmid and flanked by 25-bp border sequences in a repeated orientation. The desired gene could be inserted between the left and right border repeats of T-DNA (Gelvin, 2003). On the other hand, the 35-kb *vir* region which is located on the Ti plasmid consists of eight operons: *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, *virG*, and *virH* (Thomashow et al., 1987). These genes encode Vir proteins (Table 2.1) in response to the specific compounds secreted by the wounded plant in order to regulate the processing and transfer of T-DNA. The *chv* regions located on the *Agrobacterium* chromosome and consist of three loci: *chivA*, *chvB* and *pscA* (Table 2.1) (Douglas et al., 1985).

Table 2.1: The role of *Agrobacterium* virulence proteins in T-DNA transfer.
(Douglas et al., 1985; Thomashow et al., 1987)

Bacterial proteins	Function in T-DNA transfer
Chromosomal virulence proteins	
ChvA, ChvB, PscA	The attachment of *Agrobacterium* to the plant cell wall.
pTi plasmid virulence proteins	
VirA and VirG	The two-component signal transduction system governing vir gene expression: VirA is a membrane bound sensor kinase; VirG is a response regulator that activates vir operons.
VirB1-11, VirD4	The type IV secretion system: transfer of T-DNA and virulence factors from *Agrobacterium* to a host.
VirC1 and VirC2	Bind to an overdrive sequence adjacent to the T-DNA right border and interact with the VirD2/D1 relaxosome.
VirD1 and VirD2	The relaxosome complex involved in the production of T-DNA: VirD2 is the main enzyme nicking theT-DNA border sequences of pTi plasmid with the help of VirD1. *In planta* functions of VirD2: the nuclear import of T-DNA and T-DNA integration.
VirE1	A VirE2 chaperone protein that regulates the association between T-DNA and VirE2.
VirE2	A single-stranded DNA binding protein that (i) forms a channel-like structure, likely facilitating the penetration of the host cell's periplasmic membrane, (ii) protects T-DNA from host nucleases, (iii) facilitates nuclear import of T-DNA via interactions with VIP1.
VirE3	A functional homologue of the host VIP1 protein; both proteins interact with VirE2 and the host nuclear import factors.
VirF	The uncoating of T-DNA by directing the host protein degradation system to VirE2 via its interaction with VIP1.
VirH2	Play a role in the metabolism of several phenolic compounds including ferulic acid.

Agrobacterium-plant cell interaction involves seven essential steps (Figure 2.1) (Sheng and Citovsky, 1996). First, *Agrobacterium* binds tightly to cell surface of host plant via cellulose filaments. The detection of phenolic compounds secreted by the wounded plant cells by VirA leads to phosphorylation of VirG, resulting in induction of *vir* gene expression. Following this, VirD2 make a nick at the T-DNA border sequences of pTi plasmid with the aid of VirD1 to produce transferrable single stranded copy of the T-DNA. The T-DNA is then coated by VirE2 to form a channel-like structure, which facilitate the penetration of the host cell's membrane and protect against nucleolytic degradation. The T-complex (T-DNA along with VirD2 and VirE2) are exported into the host cell by a VirB/D4 type IV secretion system. After entering the host cell, Vir2 and E2 interact with host cell nuclear localization signal receptors to transport T-complex through the nuclear pore. The T-DNA will integrate into the host cell genome and second strand of T-DNA will be synthesized. The gene of T-DNA will be expressed.

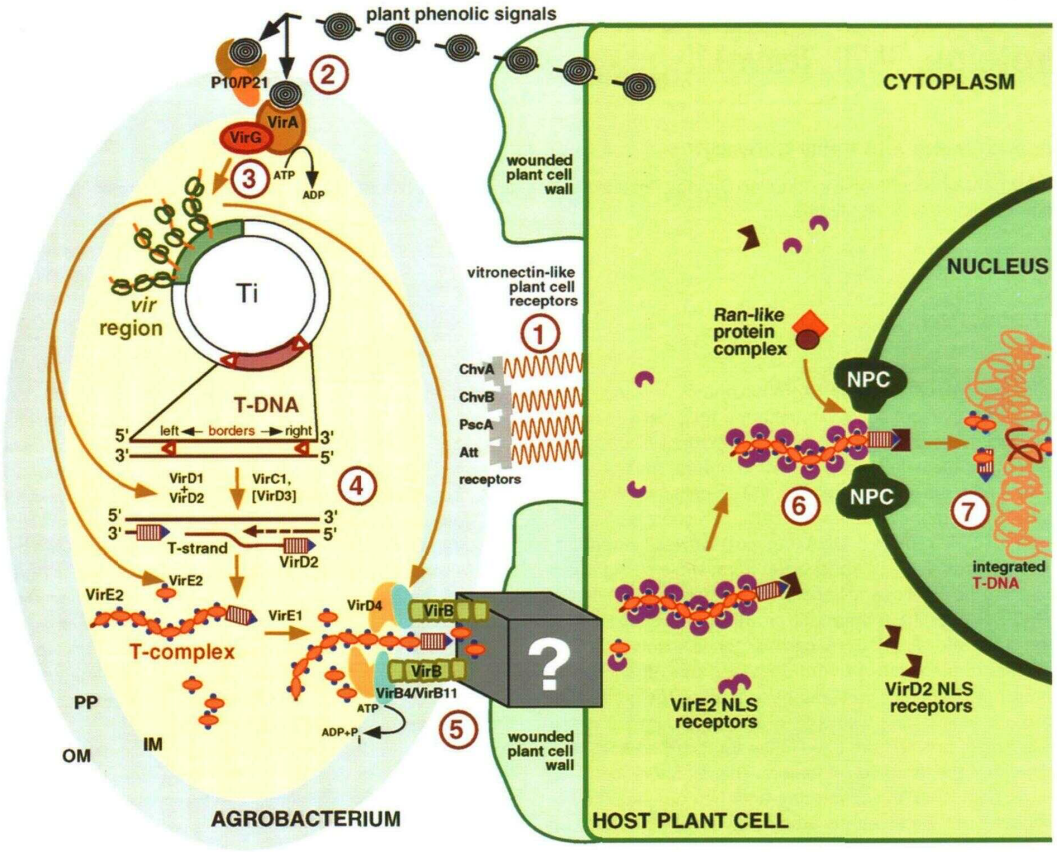

Figure 2.1: The diagram summarizes all the essential steps in the transformation of plant cells by *Agrobacterium tumefaciens*. Step 1, binding of *Agrobacterium* to the host cell surface receptors; step 2, recognition of plant signal molecules by the bacterial VirA/VirG sensor-transducer system; step 3, activation of the bacterial *vir* genes; step 4, production of the transferable T-DNA; step 5, formation of the T-complex and its transport into the host plant cell; step 6, nuclear import of the T-complex; and step 7, integration of T-DNA into host genome and expression of T-DNA. IM, bacterial inner membrane; NPC, nuclear pore complex; OM, bacterial outer membrane; PP, bacterial periplasm (Sheng and Citovsky, 1996).

Agrobacterium-mediated transformation method is cost effective because does not require sophisticated equipment. This method is also less labor intensive as the preparation of protoplast is not required. In addition, this transformation method results in a high frequency of stable genomic integration with single or low copy number of the intact transgene (Cheng et al., 2004; Graves and Goldman, 1987; Jones, 2005).

However, *Agrobacterium* infection of plant tissues could result in plant tissue necrosis (Deng et al., 1995; Hansen, 2000; Pu and Goodman, 1992). In addition, this transformation method applicable only to plants which can be infected with *Agrobacterium*. Another disadvantage is that this transformation method is not applicable to chloroplast and mitochondria transformation.

2.1.2 Polyethylene Glycol (PEG)-Mediated Protoplast Transformation

Protoplasts transformation method is widely used for transient and stable expression assays to test gene constructs in plant system (Yoo et al., 2007; Zhang et al., 2011) as well as for crop improvement (Datta et al., 1990; Masani et al., 2014; Mazarei et al., 2008; Rahmani et al., 2016; Rhodes et al., 1988; Zhang et al., 2016a). Protoplasts are plant cells without the cell wall which can be made by degrading cell walls with a mixture of the appropriate polysaccharide-degrading enzymes such as cellulase and pectinase (Cocking, 1960). Protoplasts can be isolated from a wide variety of tissues and organs that include leaves, roots, shoot apices and fruits. Among these, the most frequently used tissues are mesophyll tissue of fully expanded leaves of young plants and new shoots. Since there is no cell wall around to block the passage of molecules into the cell, protoplasts are widely used for DNA transformation. The polyethylene glycol (PEG)-mediated protoplast transformation is a direct gene transfer technique established originally for tobacco protoplasts (Negrutiu et al., 1987).

To genetically transform protoplasts, the isolated protoplasts are treated with plasmid DNA and PEG solution containing calcium and magnesium ions at alkaline pH (Negrutiu et al., 1987). During the PEG treatment, the cell membrane of protoplast will be distorted by surface-tension forces due to the cell density differences between

the PEG solution and the protoplast. This will facilitate the cellular internalization of exogenous DNA via endocytosis. To regenerate the transformed protoplasts into whole plant, the protoplasts are first grown into a group of plant cells that develop into a callus. Then, callus is stimulated to produce shoot which are then rooted and develop into plant. The growth of protoplasts into callus and regeneration of shoots requires the appropriate balance of plant growth regulators in the tissue culture medium that need to be customized for plant species (Thorpe, 2007).

This transformation method has no limitations about the species of plants and the type of organelles (nucleus, chloroplast and mitochondria) to be transformed. Thus, this method can overcome the limitation associated with *Agrobacterium*-mediated transformation. In addition, it is cost-effective as no sophisticated equipment is required.

The major difficulties with this technique are the protoplast isolation and the regeneration of plants from the transformed protoplasts. It takes a long time to regenerate whole transgenic plants and there is a risk of contamination. In addition, there is a high chance of getting some somaclonal variations in the regenerated plants (Lörz and Scowcroft, 1983; Sevon et al., 1998; Yamagishi et al., 1996). The somaclonal variations are mainly caused by newly generated mutations which are triggered by the stress factors during protoplast isolation and culture, including wounding, exposure to sterilants during sterilization, incomplete tissue and imbalances of media components (Joyce et al., 2003; Sato et al., 2011; Smulders and de Klerk, 2011).

2.1.3 Particle Bombardment Method

Particle bombardment is a method by which exogenous DNA are introduced into intact plant cells and tissues via high-velocity microprojectiles (Klein et al., 1987; Sanford et al., 1987). Over the past several years, particle bombardment has progressed into a valuable tool for plant biotechnology as it allows the direct gene transfer to a wide variety of cell and tissue types. Examples of the various plant species transformed by particle bombardment can be found in Christou (1994).

The particle bombardment method begins by coating the plasmid DNA with micro-seized tungsten or gold particles via the precipitation with calcium chloride and spermidine. The DNA-coated metal particles (microcarriers) are coated on a macrocarriers, which is accelerated with air pressure and shot into target plant cells or tissues. The microcarriers have enough momentum to penentrate the cell wall and become lodged inside cells (Klein et al., 1987; Sanford et al., 1987). Inside the cells, the DNA elutes off the particles and incorporate into the chromosomal DNA of the cells for expression of gene of interest. Selectable markers are used to identify the cells that take up the exogenous DNA.

Nowadays, the most commonly used biolistic device for plant transformation is the Biolistic® PDS-1000/He Particle Delivery System (Kikkert, 1993) marketed by Bio-Rad Laboratories (Figure 2.2). This rupture disk of the device releases high-pressure helium to propel a macrocarrier sheet loaded with millions of DNA-coated metal particles (microcarriers) toward target cells or tissues (Figure 2.2). A stopping screen is used to halts the macrocarrier while the DNA-coated metal particles continue to target and penetrate the target cells or tissues.

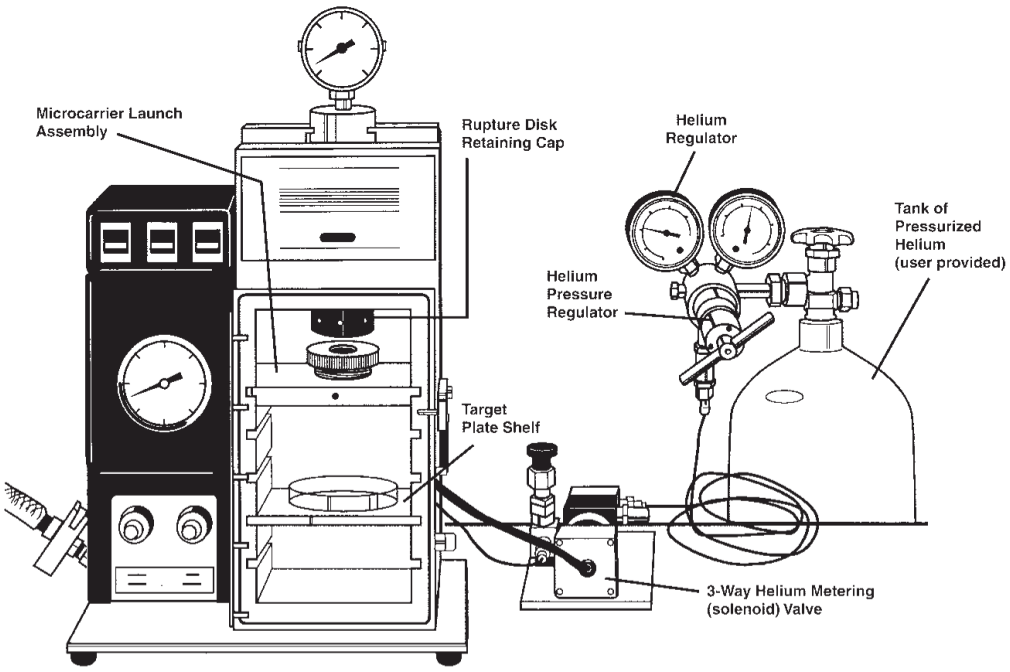

Figure 2.2: Components of the Biolistic® PDS-1000/He particle delivery system. (Drawing courtesy of Bio-Rad Laboratories, CA, USA.)(Kikkert et al., 2005).

The particle bombardment is applicable for the nuclear, chloroplasts and mitochondria transformation. It also can be used to transform all plant species. In addition, the particle bombardment involves a relatively simple and rapid protocol. The binary vector and protoplast culture are not required. Thus, this method can overcome the limitations of *Agrobacterium*-mediated transformation and difficulties associated with plant regeneration from protoplasts.

The main disadvantages of particle bombardment are the high cost of the equipment and microcarriers as well as the tissue damage caused by the uncontrollable velocity of bombardment. Another disadvantage of this method is the integration of multiple copies of transgene at single loci in the plant genome which in turn results in gene silencing (Flavell, 1994; Kohli et al., 1998; Pawlowski and Somers, 1996). Moreover, very often, incomplete sequences of the transgene are integrated into plant genome (Brettschneider et al., 1997; Walters et al., 1992).

2.2 Peptide-based Protein Delivery as an Alternative Strategy for Plant Modification

The DNA delivery is the most common method used to modify the plant due to the ease of preparation, the stability and the smaller size of plasmid DNA. Nevertheless, the DNA delivery has several drawbacks such as the random insertion of exogenous DNA into the plant genome, codon optimization and promoter selection. In light of these concerns, direct protein delivery system has emerged as a promising alternative. However, intracellular delivery of proteins is challenging because proteins have intrinsic limitations such as large molecular sizes, varying surface charges, fragile tertiary structures, susceptibility to endonuclease degradation and impermeability to cell membranes due to electrostatic repulsions (Leader et al., 2008).

The most commonly used approach for intracellular protein delivery is the use of cell-penetrating peptides (CPPs) (known as protein transduction domain) as delivery vehicle. CPPs are a group of short (at most 30–35 amino acids residues) and water-soluble peptide with ability to penetrate the cell membranes without the use of any chiral receptors and without causing significant damage to membrane. Thus, CPPs are capable of deliver covalently bound or non-covalently bound protein cargoes into the intracellular matrix with high efficiency and very low toxicity. In plant system, there is no any cytotoxic effect observed for the use of CPPs alone or in complex with their protein cargoes in somatic embryos (Chugh and Eudes, 2008b) or microspores (Chugh and Eudes, 2007) or suspension culture (Mizuno et al., 2009) or protoplast (Chugh and Eudes, 2008a) or plant tissues (Chang et al., 2007; Chang et al., 2005; Chugh and Eudes, 2008a; Lu et al., 2010). Moreover, CPPs are able to protect protein cargoes from premature degradation and various denaturing interactions with the biological environment (Faraji and Wipf, 2009).

2.2.1 Cell Penetrating Peptides

CPP was introduced in the late 1980s by the first identification of the Trans-Activator of Transcription (Tat) peptide, which is corresponding to the basic domain of Tat protein of the human immunodeficiency virus type 1 (HIV-1) (Green and Loewenstein, 1988). Several years later, penetratin peptide, which is corresponding to the third helix of the Antennapedia homeodomain of the *Drosophila melanogaster* (Derossi et al., 1994; Joliot et al., 1991) was identified. In year 2008, several new peptides were introduced: (i) naturally occurring CPPs, e.g. Herpes Simplex Virus Tegument Protein VP22 and cell wall protein-derived peptide Inv3 from *Mycobacterium tuberculosis* (Torchilin, 2008)); (ii) Chimeric CPP, e.g. transportan, a chimera of the wasp venom toxin mastoparan and neuropeptide galanin; and (iii) synthetic CPPs, e.g. model amphipathic peptide (MAP) and arginine oligomers (Jearawiriyapaisarn et al., 2008).

2.2.2 Classes of Cell Penetrating Peptides

CPPs differ significantly in their sequences, hydrophobicity and polarity. According to this, CPPs can be grouped into five categories: (i) cationic peptides, (ii) amphipathic peptides, (iii) proline-rich peptides, (iv) antimicrobial peptides, and (v) chimeric or bipartite peptides (Pooga and Langel, 2005).

Cationic CPPs comprised mainly of multiple arginine and lysine residues. Each arginine residue will exhibit positive charge at physiological pH as its guanidine side chain consists of a pKa of ~12. The guanidine side chain is postulated to interact with the negatively charged phosphates and sulfates on cell membrane surfaces via the formation of a stable bidentate hydrogen bonds. Lysine exhibit same positive charge

as arginine but does not contain the guanidine head group. The number and location of arginine and lysine residues in the sequence of cationic CPPs is a critical factor affecting the transduction efficiency of CPPs (Maiolo et al., 2005). There are various examples of arginine-rich peptides derived from known proteins, polyarginines (Futaki et al., 2001; Nakase et al.) and peptoids (Wender et al., 2000). The HIV-1 Tat basic domain (RKKRRQRRR) is a highly basic peptide which can translocate across cell membranes (Vivès et al., 1997; Wender et al., 2000). The presence of higher number of arginine residues in the dimer of the Tat peptide, Tat_2 (RKK RRQRRRRKKRRQRRR) contributes to its enhanced cell membrane penetrating ability in both animal and plant system (Chugh and Eudes, 2007; Wender et al., 2000). The penetratin, a 16-residue peptide (RQIKIWFQNRRMKWKK) derived from *Drosophila-antennapedia* transcription factor, also exhibits the translocation ability across cell membranes (Derossi et al., 1994; Deshayes et al., 2008). M918 which was derived from the C-terminus of the tumor suppressor protein p14ARF (El-Andaloussi et al., 2007) shares the cationic nature of penetratin and composed of 22 amino acids (MVTVLFRRLRIRRACGPPRVRV).

Amphipathic CPPs form amphiphilic helical structure, with all the hydrophilic residues pointing to one face and the hydrophobic residues on the opposite side (Lazar, 2004). The hydrophilic face is mainly comprised of lysine residues. The presence of amphiphilic helical structure is considered as an essential structural requirement for the cellular internalization of CPP (Scheller et al., 2000). MAP and Pep-1 are two commoly used amphipathic CPPs. MAP is an 18-residues peptide (KLALKLALKALKAALKLA) which consists of α-helical structure (Oehlke et al., 1998). Pep-1 which is a synthetic peptide consists of 21 amino acid (KETWWETWWTEWSQPKKKRKV) has been developed as a first commercial CPP

(Chariot kit, Active Motif, USA) for the non-covalent protein trasduction. Pep-1 consists of three domains: (i) a hydrophobic domain made up of multiple tryptophan residues; (ii) a hydrophilic lysine-rich domain; and (iii) a spacer domain which is responsible for the improvement of the flexibility and the integrity of both the hydrophobic and the hydrophilic domains (Morris et al., 2001).

Proline-rich peptides and polyproline-rich peptides adopt a well-defined helical structure (polyproline II) in water. The amphipathic helical structure can be formed by the introduction of polar amino acids at certain position of the peptides. One of the example of proline-rich peptide is sweet arrow peptide (SAP) (VRLPPP) which is derived from the proline-rich N-terminal repetitive domain of c-zein, a storage protein in maize. SAP consists of 50% proline sequences along with three arginine residues (Fernández-Carneado et al., 2004).

Antimicrobial peptides (AMPs) exhibit microbiocidal properties by damaging bacterial cell membranes upon cell entry and inhibiting intracellular targets. One of the common AMPs is $S4_{13}$-PV (ALWKTLLKKVLKAPKKKRKVC), a peptide results from the combination of a 13-residues derived from dermaseptin S4 peptide with the SV40 large T-antigen NLS (ALWKTLLKKVLKAPKKKRKVC) (Mano et al., 2006). Both cationic CPPs and AMPs share molecular similarity and consist of cationic peptides, which are capable of translocate across the cell membrane.

Chimeric or bipartite peptides consists of two or more of the listed motifs. The two chimeric or bipartite peptides which were internalized by various plant tissues of triticale seedlings are transportan and peptide vascular endothelial-cadherin (pVEC) (Chugh and Eudes, 2008a). Transportan is a 27-residues chimeric peptide (GWTLNSAGYLLGKINLKALAALAKKIL) that consists of 12 amino acid

sequences derived from the N-terminus of neuropeptide galanin, with a lysine residue linking the last 14 C-terminal residues of mastoparan (a peptide derived from wasp venom) (Pooga and Langel, 2005). The pVEC peptide consists of 18 amino acid (LLIILRRRIRKQAHASK) which is derived from murine pVEC [amino acid 615–632, (Elmquist et al., 2001)].

2.2.3 CPP-Mediated Delivery of Proteins in Plant System

CPP-based protein delivery system is well developed in mammalian cell system. However, CPP-mediated intracellular delivery of protein is a relatively nascent technology in plant system (Chang et al., 2005; Chugh and Eudes, 2007; Eggenberger et al., 2011; Mizuno et al., 2009). Plant cells differ from animal cells in the way that it consists of rigid cell wall surrounding the cell membrane. The pectin layers of the plant cell walls consist of free carboxylate groups of galacturonic acid moieties, lead to the high possibility that the positively charged CPPs interact with the negative charges in the cell walls (Chuah et al., 2015; Lakshmanan et al., 2013; Mizuno et al., 2009). This lead to the challenge in the peptide-based protein delivery in plant system.

CPP-mediated protein delivery in plant cells was first demonstrated for the core histones, namely H2A, H2B, H3 and H4 which was shown to penetrate plasma membranes of plant cells (*Petunia* protoplasts and cultured cells) (Rosenbluh et al., 2004). Moreover, it was demonstrated that the histones H2A and H4 were able to mediate delivery of covalently attached BSA molecules into plant cells. The results revealed the potential of the histones as CPP for the delivery of macromolecules into plant cells. In later year, peptides, such as Tat, Tat_2 (a proprietary dimer of Tat), M-Tat, pVEC and transportan, were identified to be able to trasduce protein cargoes into

wheat immature embryos (Chugh and Eudes, 2008b). Furthermore, Chugh and coworkers have demonstrated that Tat, Tat_2 and Pep-1 (commercially available Chariot kit) were able to transduce β-glucuronidase (GUS) enzyme (272 kDa) in its active form in 14% and 31% of the microspores, respectively (Chugh et al., 2009). A recent investigation is attempt to develop a CPPs that are highly stable insides cells regardless of varying salt and pH conditions. Keeping these points in view, a marine AMP derived from Japanese horseshoe crab (*Tachypleus tridentatus*), known as tachyplesin (Tpl), was designated as a CPP. Its cell-penetrating ability and cargo delivery ability were investigated in plant system, both monocot and dicot (mesophyll protoplasts and tissues of *Triticum aestivum* and *Brassica napus*) (Jain et al., 2015). It consists of 17 amino acids and a net positive charge of +7. Its structure constitutes of two antiparallel β sheets linked together by two disulfide linkages which are formed by the four cysteine residues. Apart from the studies conducted by Chugh and coworkers (2008 and 2009), another research group has reported the covalent and noncovalent transduction of 24 kDa fluorescent reporter proteins in mung bean, soybean, corn, and onion root tip cells, with the aid of Tat and arginine-rich intracellular delivery (AID) peptides (Chang et al., 2007; Chang et al., 2005; Lu et al., 2010; Wang et al., 2006).

2.2.4 BP100

BP100 (KKLFKKILKYL-amide) is an antimicrobial peptide which was developed by systematic mutation of Pep3 (WKLFKKILKVL-NH_2), a chimeric peptide derived from the naturally occurring cecropin-A (an antimicrobial peptide from the moth *Hyalophora cecropia*) and melittin (a membrane-permeabilizing component of bee venom) (Ferre et al., 2006; Ferre et al., 2009). It exhibits high

antimicrobial activity against plant pathogens such as *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas vesicatoria*. A study revealed that BP100 forms an amphiphilic α-helix in the presence of membranes: one face of the helical structure consists of hydrophilic Lys residues, while all hydrophobic residues are on the opposite side (Wadhwani et al., 2014). It kills the bacteria by permeabilizing bacterial cell membranes via formation of cationic amphiphilic structures (Boman, 2003). On the other hand, a study demonstrated that BP100 is a fast and efficient cargoe carrier to deliver functional cargoes into tobacco cells (Eggenberger et al., 2011). Thus, BP100 is a genuinely multifunctional peptide which acts on one hand as an antimicrobial membrane-permeabilizing agent, and on the other hand as an efficient cargo carrier without causing leakage in eukaryotic cells or causing significant toxicity.

2.3 Formation of Peptide-Protein complexes

There are two approaches that can be used to couple CPPs with protein cargoes: covalent protein transduction (CPT) and non-covalent protein transduction (NPT)**.** The majority of earlier studies used CPT, but the number of studies using NPT is increasing rapidly.

2.3.1 Covalent Protein Transduction (CPT)

The covalent protein transduction strategy involves the formation of a covalent conjugate between the cargo and the CPP, via chemical conjugation or via cloning and expression of a CPP fusion protein (Zatsepin et al., 2005). Most of the works have been reported for peptides such as Tat, penetratin, polyarginine peptide Arg8 sequence,

transportan, VP22 protein derived from herpes simplex virus, antimicrobial peptides Buforin I and SynB as well as SAP (Murriel and Dowdy, 2006; Pujals et al., 2006).

CPP-mediated CPT in plants was first demonstrated by the delivery of CPP-fusion proteins such as GFP, red fluorescent protein (RFP) and enzymes into various types of plant cells in active forms (Chang et al., 2005). The covalent-transduction of Tat-GFP, R9-GFP and R9-RFP proteins was neither species-specific nor tissue-specific in the plants studied. The major disadvantage of CPT is that it requires relatively expensive and labor-intensive synthesis of CPP fusion protein. Moreover, CPT has the risks of altering the biological activity of the cargo. Thus, the number of studies using non-covalent protein transduction (NPT) is increasing.

2.3.2 Non-Covalent Protein Transduction (NPT)

Non-covalent strategy requires only the mixing of CPP and cargo to form a complex and the bonding involve diverse variations of van der Waals forces, electrostatic interactions and hydrophobic effects. This strategy involves the use of short amphipathic peptide carriers. The amphipathic peptides consist of both hydrophilic (polar) domain and hydrophobic (non-polar) domain. There are two types of amphipathic peptides: (i) primary amphipathic peptides, which are composed of a sequential assembly of hydrophobic and hydrophilic residues; and (ii) secondary amphipathic peptides, which are formed by the conformational state that allows positioning of hydrophobic and hydrophilic residues on opposite sides of the molecule (Morris et al., 2008).

The common primary amphipathic peptides, Pep-1 and MPG, consist of same hydrophilic domain but different hydrophobic domain. Both of their hydrophilic