OPTIMIZATION OF NUCLEIC ACID DELIVERY VIA CATIONIC POLYMERS FOR GENOME ENGINEERING

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ABSTRACT

OPTIMIZATION OF NUCLEIC ACID DELIVERY VIA CATIONIC POLYMERS FOR GENOME ENGINEERING

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One of the most challenging aspects of genome engineering is the delivery of genome editing components such as plasmids, oligonucleotides, RNA and protein. In this work, in-house synthetized cationic polymer poly (2-hydroxypropylene imine) (pHP) was tested in order to achieve substantial delivery efficiency while preserving high culture viability. Applicability of this cationic polymer mediated nucleic acid delivery method for both plant and mammalian cells were demonstrated. Several parameters of plasmid and oligonucleotide delivery were optimized. It was demonstrated that the working concentration of the synthesized pHP was 2 mg/mL for maize protoplasts and 10 mg/mL for HEK239 cell line. Most crucial parameter for plasmid delivery was DNA to polymer ratio. For oligonucleotide delivery, it was discovered that if the polyplex sizes kept smaller, the delivery efficiency increased. Additionally, it was shown that increased amounts of oligonucleotides and polymer have adversely affected culture viability. Finally, CRISPR/Cas9 and Oligonucleotide Targeted Nucleotide Exchange (OTNE) mediated genome editing by delivering the editing components via cationic polymer was investigated. Successful CRISPR/Cas9 mediated knockout of genomic GFP gene was demonstrated in plant cells. Use of this polymer as a mediator of nucleic acid delivery to plant cells may have further applications in genome engineering in hard to transform plant cells.

Keywords: Genome engineering, cationic polymer, plant transformation, CRISPR/Cas9, Oligonucleotide Targeted Nucleotide Exchange

ÖZ

GENOMİK DÜZENLEME İÇİN NÜKLEİK ASİTLERİN KATYONİK POLİMERLER İLE İLETİLMESİNİN OPTİMİZASYONU

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Genom mühendisliğinin en zorlu yönlerinden biri, plazmit, oligonükleotit, RNA ve protein gibi genom düzenleme bileşenlerinin hücrelere iletilmesidir. Bu çalışmada laboratuvarımızda sentezlenen katyonik polimer poli (2-hidroksipropilen imin) (pHP), kültürdeki hücrelerin canlılığını korurken yeterli miktarda nükleik asit molekülünün iletilip iletilmediğini görmek üzere test edildi. Katyonik polimer ile nükleik asit iletme yönteminin bitki ve memeli hücreleri için uygulanabilirliği gösterildi. Çeşitli plazmit ve oligonükleotit iletme parametreleri optimize edildi. Sentezlenen pHP'nin çalışma konsantrasyonunun mısır protoplastları için 2 mg/mL ve HEK239 hücre hattı için 10 mg/mL olduğu gösterildi. Plazmit iletimi için en önemli parametrenin DNA/polimer oranı olduğu keşfedildi. Oligonükleotit iletimi için eğer polipleks boyutları daha küçük tutulursa, iletim verimliliğinin arttığı keşfedildi. Ek olarak, artan miktarda oligonükleotitlerin ve polimerin kültürdeki hücrelerin canlılığını olumsuz yönde etkilediği gösterildi. Son olarak, CRISPR/Cas9 ve Oligonukleotit Hedefli Nükleotit Değişimi (OHND) ile genomik düzenlemenin bileşenlerin katyonik polimer ile iletilmesi araştırıldı. Bu çalışmada, CRISPR/Cas9 sistemi ile genomik GFP geninin işlevsiz hale getirilebildiği gösterildi. Bu polimerin nükleik asit iletiminde kullanılması zor transforme olan bitki hücrelerinde genom mühendisliği alanında farklı uygulamalara olanak sağlayacaktır.

Anahtar Kelimeler: Genom düzenleme, katyonik polimer, bitki transformasyonu, CRISPR/Cas9, Oligonukleotit Hedefli Nükleotit Değişimi

To my family

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
A5	Transgenic maize cell line containing fuctional copy of GFP; transformed via *A. Tumefaciens*
ANOVA	Analysis of Variance
B71	Transgenic maize cell line containing fuctional copy of GFP; transformed via particle bombardment
Cas9	CRISPR Associated Protein 9
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR Activation
CRISPRi	CRISPR Interference
cv.	Cultivated Variety
Cy3	Cyanine 3
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
FAM	Fluorescein
FBS	Fetal Bovine Serum
GFP	Green Fluorescence Protein
GFP7	Transgenic maize cell line containing mutant version of GFP

H1233	Non-transgenic maize cell line
HDR	Homolgy Directed Repair
HEK239	Human Embryonic Kidney 293 Cell Line
HeLa	Henrietta Lacks (uterine cell variety; named for deceased patient)
mGFP	GFP gene with premature stop codon
mRNA	Messenger RNA
NHEJ	Non-Homologous End Joining
OTNE	Oligonucleotide Targeted Nucleotide Exchange
PCV	Packed Cell Volume
PEG	Polyethylene Glycol
PEI	Polyethyleneimine
pHP	Poly(2-hydroxypropylene imine)
PLL	Poly-L-lysine
ppIS	Plant Protoplast Isolation Solution
PPT	Phosphinothricin
PTO	Phosphorothioate
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNP	Ribonucleoprotein Complex
rpm	Revolution per minute
SD	Standar Deviation
sgRNA	Single Guide RNA

ssODN	Single-Stranded Oligodeoxynucleotides
ssp.	Species
TALEN	Transcription Activator-Like Effector Nuclease
ZFN	Zinc Finger Nuclease

CHAPTER 1

INTRODUCTION

1.1. Genome Editing Methods

1.1.1. Oligonucleotide Targeted Nucleotide Exchange (OTNE)

OTNE is a targeted gene repair system that utilizes the endogenous repair machinery of the cell to change a target nucleotide. This method is not used to introduce novel sequences to the genome, rather it aims to change an existing nucleotide in the genome to mutate or correct a gene. It is believed that there are 2 steps involved in this genome editing method (Figure 1.1). The first step is base pairing between oligonucleotide template and its complementary sequence on the genomic DNA. In the second step, this oligonucleotide/DNA duplex is recognized by endogenous DNA repair machinery, which then facilitates the base exchange.

There are several documentations of successful OTNE mediated genome editing in literature. Especially for plants, this method was utilized to generate herbicide-resistant cultivars (Zhu et al., 2000; Kochevenko et al., 2003). However, OTNE method suffers from low efficiency, difficulty of selecting edited cells and off-target effects. Various approaches were tested in order to address these issues. Most notable ones include using DNA damage agents such as hydroxyurea and the etoposide VP16 (Ferrara et al., 2004) to increase the frequency of OTNE events. Another approach was to reduce genomic compaction with histone deacetylase inhibitors, which increased OTNE efficiency in plants (Tiricz et al., 2018).

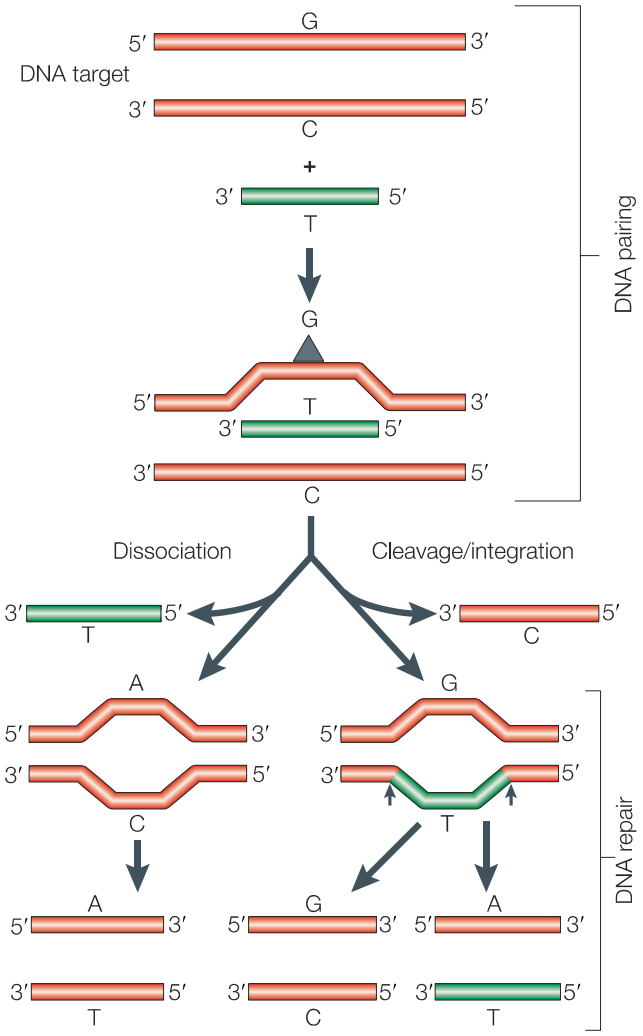

Figure 1.1. Mechanism of OTNE mediated genome editing

(Figure taken from Liu et al., 2003)

1.1.2. Nuclease Mediated Genome Editing

High efficiency genome editing has become possible with the discovery of engineered nucleases that can target specific sequences on the genome. These nucleases introduce double-strand breaks (DSBs), which alert the DNA repair machinery.

There are two general repair pathways that can occur upon introduction of DSBs: non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ pathway is error-prone and can cause frame-shift mutations, which means that this pathway can

be used for gene knockouts. For HDR, a correction template should be present. The endogenous repair machinery uses this template to correct the region around DSB. This pathway can be exploited if a correction template with the desired sequence is introduced to the cell (Figure 1.2). Correction templates can be double-stranded DNA with homology arms or single-stranded oligodeoxynucleotides (ssODNs) (Cong et al., 2013; Ran et al. 2013).

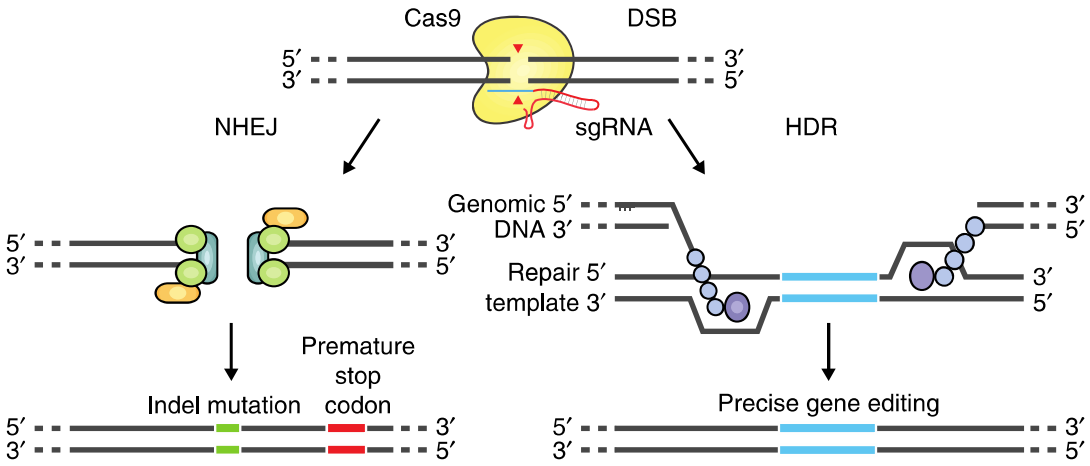

Figure 1.2. Endogenous repair pathways induced upon DSB introduction by Cas9.

(Figure taken from Ran et al. 2013)

1.1.2.1. Zinc Finger Nucleases (ZFNs)

Initial genome editing approach was to design modular proteins that contain a DNA-binding domain fused with an effector domain. In this method, the DNA-binding domain is used to target the modular protein to a specific target sequence on the genome. This domain is typically derived from naturally occurring DNA-binding proteins such as zinc finger proteins and transcription activator-like effectors (TALEs). The effector domain can be selected according to the researchers purpose. For genome editing, DNA-binding domain can be fused to the catalytic domain of an endonuclease in order to introduce double strand break (DSB) to the region of interest. Fusion of the DNA-binding domain with transcription factors, methyltransferases and

recombinases is also possible, thus allowing different modifications in the selected genomic region.

ZFNs were the first nuclease based genome editing method described (Kim et al., 1996). The Cys2–His2 zinc finger domain recognizes approximately 3 bases of DNA. By engineering the residues that form the alpha-helix motif, DNA-binding specificity can be modified. Thus, researchers were able to identify various zinc finger domains that have binding specificity to almost all possible 3 base combinations (Perez-Pinera et al., 2012). Additionally, several zinc finger motifs can be conjugated in order to target unique sequences on the genome.

1.1.2.2. Transcription Activator-Like Effector Nucleases (TALENs)

TALEs were identified as DNA-binding proteins of plant pathogenic bacteria (Moscou et al., 2009). They have 34 amino acids that recognize a single base pair. DNA binding specificity depends on two hypervariable amino acids in 12^{th} and 13^{th} positions called as repeat-variable di-residue (RVD). Similar to zinc finger domain, modifying these residues change the DNA binding specificity and allow researchers to alter the target sequence of the protein. Moreover, several TALE repeats can be conjugated to target more specific regions on the genome (Bogdanove et al., 2011).

Catalytic domain of the Type IIS restriction endonuclease FokI is the most commonly used effector domain. Due to the nature of FokI nuclease, a pair of ZFNs or TALENs needs to be designed in order to cleave the target sequence. This provides higher specificity and decreases off-target events (Urnov et al., 2010). One of these ZFNs or TALENs should target the sequence on the forward strand and the other one should target the sequence on the reverse strand, flanking the target site. This allows dimerization of FokI catalytic domains (Figure 1.3). Subsequently the DNA is cleaved on both strands, generating a double-strand break (DSB) with 5′ overhangs (Gupta et al., 2014).

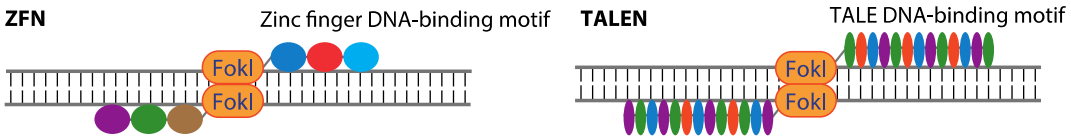

Figure 1.3. FokI dimerization in ZFN and TALEN systems.

(Figure taken from Wang et al., 2016)

Designing functional nucleases with high specificity to target region is challenging. Required labor and expertise in construction of ZFNs and TALENs is the greatest disadvantage of these systems (Hsu et al., 2014).

1.1.2.3. CRISPR/Cas9 System

The first CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) array was discovered in *Escherichia coli* in 1987, however it took almost 20 years until researchers could identify the function of these arrays (Ishino et al., 1987; Barrangou et al., 2007). As the name suggests, CRISPR arrays consist of repeats, spacers, leaders and CAS (CRISPR associated) genes (Figure 1.4a). Although these vary between different microbial species, there are some common features. For instance, in a given CRISPR array, the length and sequence of the repeats were found to be identical and some repeats contained short palindromic sequences. On the other hand, spacer sequences are unique in each CRISPR array and it was shown that they are derived from phage DNA to provide acquired resistance to viral infections (Figure 1.4b-c) (Sorek et al., 2008).

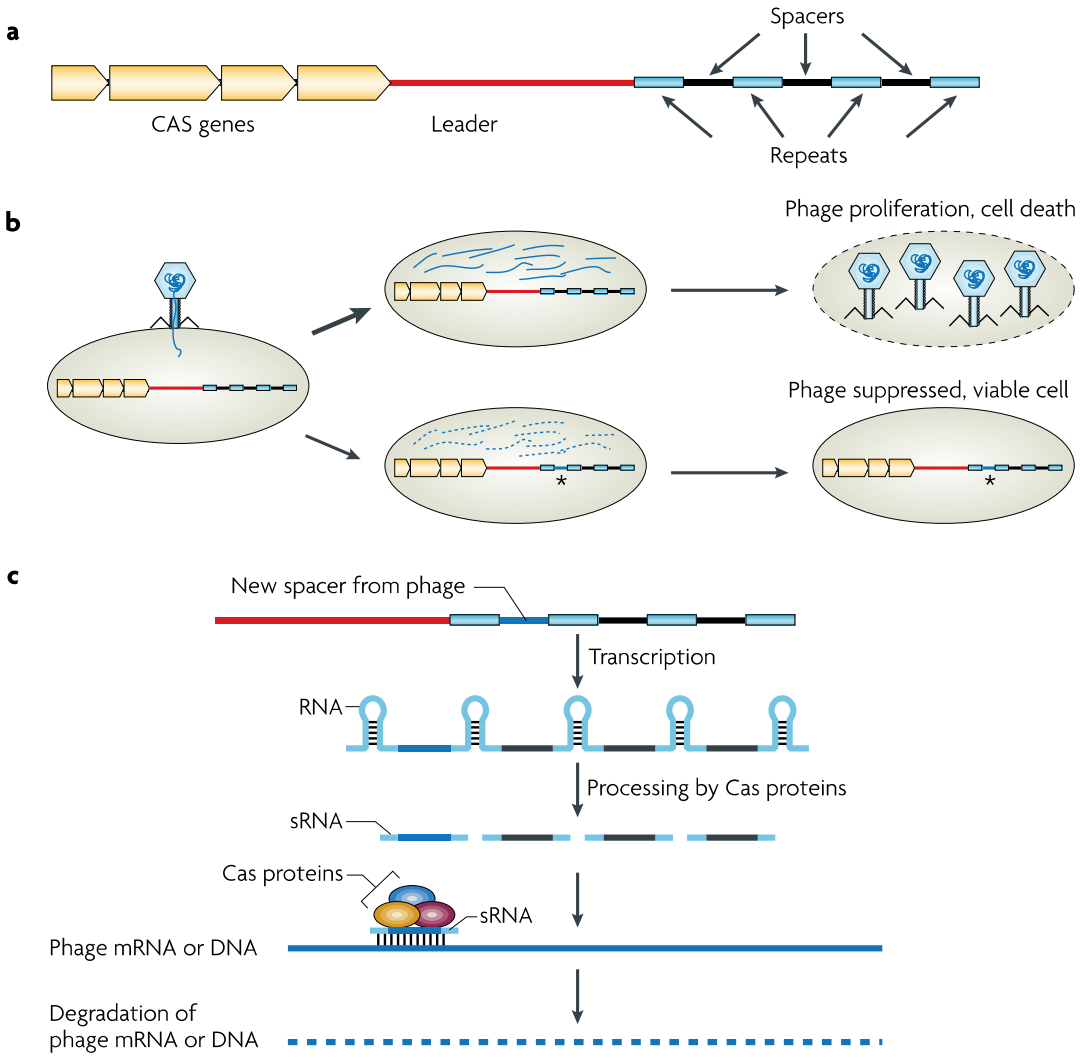

Figure 1.4. The CRISPR array.

a. CRISPR array consists of CAS genes, Leader, Repeat and Spacer regions. b. Upon phage infection, bacteria can survive when a part of the phage genome is inserted into the CRISPR array. c. Mechanism of adaptive immunity provided by the CRISPR/Cas system. (Figure taken from Ran et al., 2013)

The Type II CRISPR system from *Streptococcus pyogenes* is the most commonly used system in genome editing. It consists of the endonuclease Cas9 and two short RNA molecules CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). crRNA is transcribed from the spacer region and contains a 20 nucleotide long guide sequence as well as a short repeat sequence that allows base pairing with tracrRNA. In the originally identified system, both crRNA and tracrRNAs are required for Cas9 to be

guided to the target sequence. However, a single guide RNA (sgRNA) formed by the fusion of tracrRNA and crRNA is also sufficient to guide Cas9 to a 20-nucleotide target sequence (Jinek et al., 2012). Later, CRISPR/Cas9 system was adapted as a precise genome-editing tool (Cong et al., 2013; Ran et al. 2013). This system allowed researchers to target the endonuclease Cas9 to introduce DSB to virtually any region on the genome by designing a short sgRNA. High editing efficiency and ease of use attracted many researchers, thus the utilization of CRISPR/Cas9 system for genome editing became highly prevalent (Adli, 2018).

After the adaptation of CRISPR/Cas9 system for genome editing, researchers began to explore new application possibilities (Figure 1.5). Although there are many such applications of this system, only the major and well-established ones will be mentioned here.

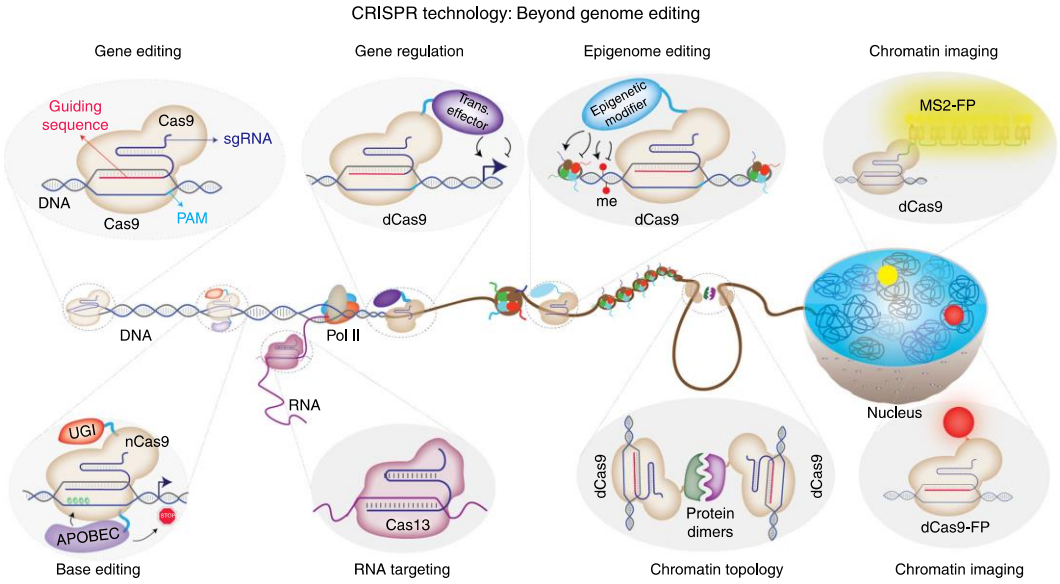

Figure 1.5. Various application areas of the CRISPR/Cas system

(Figure taken from Adli, 2018)

Wild-type Cas9 endonuclease contains two nuclease domains. HNH domain cleaves the strand that is complementary to the sgRNA and RuvC domain cleaves the other strand. Nickase Cas9 (nCas9) is generated by mutating one of these nuclease domains.

Instead of introducing DSBs, nCas9 cuts only one of the DNA strands. Thus, two nCas9s can be targeted to adjacent DNA sites to introduce DSB, which is used to increase specificity. When both nuclease domains are mutated the protein is called as dead Cas9 (dCas9). It lacks the ability to cut the DNA, however it can still be guided to the target region with sgRNA. dCas9 can be fused to various effectors to be used in different applications (Wang et al., 2016).

The nCas9 can be used for precise base editing such as conversion of cytosine to thymine or adenine to guanine. Such conversions can be achieved when nCas9 is fused to deaminase enzymes. It was shown that nCas9 fused with APOBEC1 deaminase and uracyl glycosylase inhibitor (UGI) could convert cytosine to thymine in the target sequence. Similarly, adenosine to thyrosine conversion was achieved by fusing nCas9 with a transfer RNA adenosine deaminase (Komor, et al., 2016; Gaudelli, et al., 2017). Consequently, the CRISPR-STOP approach was defined as manipulating the genetic code to introduce early stop codons. This method provides high efficiency with lower side-effects when compared to wild type Cas9-mediated gene knockout approach (Adli, 2018).

Cas9 can also be repurposed for sequence-specific gene regulation. One such method is CRISPR interference (CRISPRi) approach. In this system, binding of dCas9 to the target sequence interferes with transcription of that gene, resulting in gene silencing. Additionally, if a transcription repressor complex such as Kruppel-associated box (KRAB) is fused with dCas9, an enhanced gene silencing can be achieved. On the other hand, CRISPR activation (CRISPRa) approach enables enhanced expression of the target gene by fusing dCas9 to transcriptional activators, such as VP64 and p65AD (La Russa et al., 2015). CRISPRi/a method is more advantageous than other gene expression regulation methods such as RNAi and TALE/ZF since it is very simple to design and provides high specificity (Wang et al., 2016).

One of the most exciting application fields of the CRISPR/Cas9 system is epigenome editing. dCas9 can be fused with various histone modifiers and DNA methylases/demethylases to change the epigenetic status of the target region. For instance, dCas9 was fused to the histone demethylase LSD1, which removes

H3K4me2 marks (Kearns et al., 2015). Such fusion Cas9 proteins can be used for investigating the connection between certain epigenetic marks with expression profiles.

Another application area of modified Cas9 proteins is chromatin imaging. Fluorescent in-situ hybridization (FISH) method was routinely used for this purpose, however it is not possible to use FISH for live cell imaging since it requires fixation of the cells. Fluorescently labeled dCas9 can be targeted to virtually any genomic region of interest for visualization of the genomic region in live cells. Both repetitive and non-repetitive regions on the genome can be targeted by designing sgRNAs accordingly.

The CRISPR/Cas9 system can be used for large-scale genome-wide functional screenings. For this purpose hundreds of sgRNAs should be used in a cell population. A sgRNA library can be generated to target Cas9 to gene coding regions, thus functions of certain genes in various biological processes can be identified. This provides better insight about the gene functions than RNAi-mediated functional screenings since knockout of the target genes rather than knockdown leads to more significant phenotypic changes (Wang et al., 2016).

In order to achieve CRISPR/Cas9 mediated genome editing in a given cell, functional Cas9-gRNA ribonucleoprotein (RNP) complexes should be present in the cells. There are several ways to introduce these components to cells. Both Cas9 protein and gRNA coding plasmids can be used to produce these biomacromolecules within the cells. Another approach is to present Cas9 encoding mRNA molecules along with sgRNA molecules. Finally, Cas9 and gRNA can be presented as ready-to-use RNP complexes (Liang et al., 2015).

1.2. Plant Transformation Methods

1.2.1. Agrobacterium-Mediated Gene Transfer Method

The soil bacterium *Agrobacterium tumefaciens* is known to infect a wide range of dicotyledonous plants. In nature, this phytopathogen inserts the T-region of its Ti plasmid into the genome of the host plants and consequently causes crown gall

disease. Researchers have exploited this feature of *A. tumefaciens* to transfer gene of interest into the plant cells by inserting this foreign DNA sequence within the T-region of the Ti plasmid (Herrera-Estrella et al., 1983). For Agrobacterium-mediated gene transfer, actively dividing tissues such as callus tissue are used. The cells are co-cultured with Agrobacterium and treated with acetosyringone, to induce Agrobacterium genes that are required for gene transfer (Komari et al., 1998).

Agrobacterium-mediated gene transfer is a routinely used protocol for transformation of plants even today. Certain advantages of this method include integration of intact foreign DNA fragments and high fertility of the transgenic plants. However, there are certain downsides of this technique that limits its application. Most importantly, copy number of the inserted gene is very low when compared to other transformation methods such as particle bombardment (Dai et al., 2001). Another noteworthy drawback is the difficulty of getting rid of the bacterial infection.

1.2.2. Physical Delivery Methods

Physical methods include particle bombardment, electroporation and electrophoresis. Common feature of these methods is that they facilitate biomacromolecule delivery via deforming the cell membrane either by mechanical force or electric field. Hence, these techniques do not require a vector to deliver the biomacromolecules.

1.2.2.1. Particle Bombardment

Particle bombardment or biolistics was described as a universal method for plant transformation (Sanford et al., 1987). Biological materials such as DNA and RNA can be precipitated onto tungsten or gold microparticles. These microprojectiles are accelerated in the particle gun, consequently they penetrate intact tissues or cells. Particle bombardment became a staple in plant transformation research due to its universal applicability, relatively higher transformation efficiency and the ability to transform intact tissues and cells containing cell walls. Due to the nature of this method, researchers should obtain a particle gun to conduct experiments, which is a disadvantage when compared to some other methods that do not require any additional equipment.

1.2.2.2. Electroporation

Electroporation method uses electrical fields to transiently destabilize the membrane allowing the entry of normally impermeable macromolecules into the cytoplasm (Fromm et al., 1985 & 1986). This method was shown to be effective for both plant protoplasts and intact tissues. However, it is not a commonly used method for plant transformation.

1.2.2.3. Electrophoresis

Electrophoration of immature embryos was developed as an alternative method of plant transformation. This method uses two pipette tips, where one of them is the anode and the other one is the cathode. Both tips are filled with agarose but only the cathode tip contains DNA mixed in the agarose. The embryo is placed between two tips and electric current is applied. Subsequently, DNA migrates through cathode to anode. Note that cathode is connected to apical meristem, thus DNA enters the meristemic tissue first (Rakoczy-Trojanowska, 2002). Electrophoresis mediated transformation is also an uncommon method, probably due to its complexity to set-up.

1.2.3. Chemical Delivery Methods

One of the most common methods for introducing DNA to plant protoplasts is PEG-induced direct DNA uptake (Mathur et al., 1998). In early studies, PEG treatment was shown to enhance direct DNA uptake in *Nicotiana tabacum* (Negrutiu et al., 1987). Later, this method was applied for several different plant species (Maas et al., 1989; Hayashimoto et al., 1990; Lazzeri et al., 1991).

Transformation efficiencies up to 60% (Abel et al., 1994) and 90% (Yoo et al., 2007) after PEG-induced direct DNA uptake has been reported for plants but achieving such high transformation efficiencies require intensive optimizations. On the other hand, protoplast fusions caused by PEG treatment can be a drawback if mitotic activity of the protoplast culture after transformation is important. PEG is widely used for plant protoplast fusion experiments (Reinert et al., 1982) but fusions are not desirable in case of nucleic acid delivery. Although spontaneous fusions occur in maize protoplast

cultures (Brar et al., 1979), it has been demonstrated that chemically induced-fusion leads to larger multinuclear protoplast formation at higher frequencies when compared to spontaneous fusion (Withers et al., 1972). Therefore, mitotic activity can get adversely affected by chemically induced protoplast fusions. This is especially an inconvenience for genome editing techniques that require cell divisions such as OTNE or if callus or full plant is desired for further experiments and validations.

1.2.4. Nanomaterial-based Delivery Methods

1.2.4.1. Lipid-based Nanomaterials for Nucleic Acid Delivery

Liposomes are hallow vesicular structures formed by mono or bilayer of phospholipids. They can be loaded with a great variety of molecules, including pharmaceutical chemicals and nucleic acids. Liposome-mediated delivery of such cargos have been extensively used in animal tissue culture as well as *in vivo* systems.

Delivery of nucleic acids via liposomes have been investigated for plant protplasts with the aid of PEG (Deshayes et al., 1985). However, this method is still far from being routine in plant transformation.

1.2.4.2. Polymer-based Nanomaterials for Nucleic Acid Delivery

1.2.4.2.1. Cationic Polymers

Cationic polymer mediated nucleic acid delivery is preferred due to its high delivery efficiency and membrane-perturbing activity in mammalian cell cultures (Boussif et al., 1995; Dehshahri et al., 2013; Pandey et al., 2016). Poly-L-lysine (PLL) and polyethyleneimine (PEI) are some of the commonly used cationic polymers for transfection experiments. Cationic polymers and negatively charged nucleic acids interact through van der Waals interaction to form polymer/DNA complexes (polyplexes). Polyplexes that attach to cell membrane are internalized via endocytosis and enter into endosomes. For successful gene editing, delivered nucleic acids should escape the endosome before endosome maturation or they will be destined for degradation (Lin et al., 2012). Cationic polymers such as PEI that have membrane-perturbing activity are better suited for gene editing studies since according to the proton sponge hypothesis, they cause osmotic swelling and rupture of endosomes,

allowing the nucleic acids to escape endosomes and enter into cytoplasm (Akinc et al., 2005; Farrell et al., 2007). Nucleic acid dissociation from polyplexes can occur before or after nuclear entry, consequently protecting nucleic acids from degradation (Dean et al., 2005). It was shown that polypex migration towards nucleus is not through random diffusion. Rather, they are transported on microtubules, which results in polyplex accumulation in perinuclear region (Suh et al., 2003). Thus, this provides an advantage of cationic polymers over cationic lipids since cationic polymers enhance DNA delivery into nucleus unlike cationic lipids (Pollard, 1998).

Many new cationic polymers have been synthesized and tested for their ability to deliver nucleic acids into mammalian cells. Poly(2-hydroxypropylene imine) (pHP), which was obtained as a result of polycondensation reaction of 1,3-diamino-2-propanol and dibromide, was shown to be as efficient in mammalian cell transfection as various commercially available transfection reagents (Zaliauskiene et al., 2010). However, applications of such cationic polymers are not explored for plant cells.

1.3. Aim of the Study

The main limiting factor in genome engineering is inefficient delivery of genome editing components to the cells. The objective of this study was optimization of plasmid and oligonucleotide delivery conditions via in-house synthesized cationic polymer pHP. Applicability of this polymer for mammalian and plant cells and CRISPR/Cas9 or OTNE mediated genome editing by delivering the editing components with pHP was aimed to be investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

All chemicals used in this study were molecular biology grade.

Cationic polymer pHP was synthetized by Dr. Györgyi Ferenc (Laboratory of Nucleic Acid Synthesis, BRC, Szeged, Hungary). Methanol and dimethyl acetamide (DMAC) were used as solvents. Synthetized polymer was dialyzed against water and lyophilized by Dr. Elfrieda Fodor (Institute of Biochemistry, BRC, Szeged, Hungary). 10 mg/mL stock solution was prepared by dissolving the lyophilized polymer in sterile double distilled water.

2.1.2. Buffers and Solutions

All information about buffer/solution compositions and preparations are provided in Appendix A.

2.1.3. Cell Lines and Cell Cultures

2.1.3.1. Plant Cultures

Zea mays H1233 suspension culture was cultured in liquid N6M culture medium supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). *Zea mays* GFP7 cell line, which contains mutant GFP gene (Tiricz et al, 2018) was cultured in liquid N6M culture medium supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 20 mg/L phosphinothricin (PPT). *Arabidopsis thaliana* Col. Gif. W. suspension culture was cultured in MM1 culture media and *Oryza sativa* L. ssp. Japonica cv. 'Unggi 9' suspension culture was cultured in G1 media (Kotogány et al., 2010). Detailed compositions of all growth media are provided in Appendix A.

2.1.3.2. Mammalian Cell Lines

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4500 mg/L), with glutamine (Biological Industries) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) Penicillin/ Streptomycin mixture.

2.1.4. Oligonucleotides

Native, Cy3 and FAM conjugated fluorescent ssODNs were synthetized by Dr. Györgyi Ferenc (Laboratory of Nucleic Acid Synthesis, BRC, Szeged, Hungary). All oligonucleotide sequences are provided in Appendix B.

2.1.5. Plasmids

pFGC-pcoCas9 and pZmU3-gRNA plasmids were obtained from Addgene. pSUPER.retro.neo+GFP vector was obtained from oligoengine™. All plasmid maps are provided in Appendix C.

2.2. Methods

2.2.1. Plant Protoplast Isolation

Maize protoplasts were isolated using protoplast isolation solution (ppIS) (Appendix A). 50 µL packed cell volume (PCV) of suspension culture was washed twice with N6M culture medium. The clusters were allowed to settle for 30 minutes before removing the medium. The frozen ppIS was thawed at room temperature. 1 mL of enzyme solution was put onto washed cells and the whole mixture was transferred into a 35 mm petri dish. Cells were incubated overnight in dark while shaking at 50 rpm. After overnight protoplast isolation, protoplasts were visualized under the microscope (Figure 2.1b). Protoplasts were washed twice with R45 medium (Appendix A).

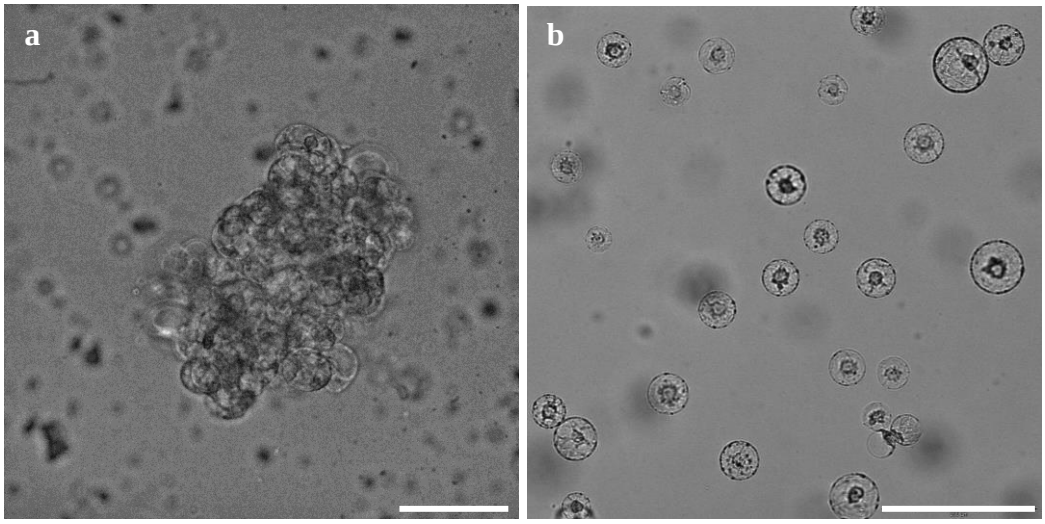

Figure 2.1. Protoplast isolation from plant suspension cultures.

a. Plant cell clusters in the suspension culture were incubated overnight with the ppIS. Scale bar 50 μm. b. Isolated protoplasts were visualized under the microscope. Note that protoplasts lack cell wall, thus they have a round shape. Scale bar 200 μm.

Haemocytometer was used to count the protoplast amount used per each sampling point (3 μL packed cell volume (PCV)). 3 μL PCV corresponded to ~15000 cells.

2.2.2. pHP Mediated Transformation of Plant Protoplasts

Protoplasts in R45 medium were transferred into an 8-well plate (surface area 0.8 cm^2/well, working volume 0.2 mL) as protoplast-dense drops approximately 20-50 μL in volume. Nucleic acid solutions were prepared in 1.5 mL tubes containing 25 μL R75 medium (Appendix A). Polymer was added onto the nucleic acid solution and the mixture was immediately completed to 200 μL by the addition of 175 μL R75 medium. This polyplex mixture was transferred onto protoplasts. Protoplasts were incubated with polyplexes for 30-60 minutes. After this incubation, 250 μL protoplast culture media (ppN6M) was added in the wells. Protoplasts were allowed to settle at room temperature for 30 minutes, then 200 μL supernatant was removed and 200 μL fresh ppN6M was added onto the wells. This step was repeated once more.

2.2.3. pHP Mediated Transfection of Mammalian Cells

Cells were grown in a 48-well plate. When the cells reached 60-80% confluency, media on the cells were removed prior to polyplex preparation so that 100 µL media remained. 1 µg DNA was added into 1.5 mL tubes containing 100 µL 0.15 M NaCl. Polymer was pipetted to the tube wall and tubes were immediately vortexed. Mixture was incubated for 30 minutes to enable polyplex formation. 100 µL mixture was transferred into each well of the 48-well plate. Polyplexes were incubated with the cells overnight.

2.2.4. PEG-Induced Direct DNA Uptake in Maize Protoplasts

After overnight protoplast isolation, protoplasts were transferred into round bottom 2 mL tubes and equal volume of W5 solution (Appendix A) was added. Tubes were centrifuged at 700 rpm for 4 minutes, then the supernatant was removed. Protoplasts were re-suspended in W5 solution at $2x10^5$ ml^{-1} concentration. Tubes were kept on ice for 30 minutes. Supernatant was removed and protoplasts were re-suspended in MMG solution (Appendix A) at $2x10^5$ ml^{-1} concentration. 10 µg GFP plasmid was prepared in 10 µL dH_2O and the DNA solution was added at the bottom of the wells of a 6-well plate. 10 µL PCV of maize protoplast in 100 µL MMG solution was added in the wells. 110 µL PEG-Ca solution (Appendix A) was added onto the protoplasts and mixed gently with the pipette tip. Protoplasts were incubated for 15 minutes. 400 µL W5 solution was added after the incubation to stop the reaction. After the protoplasts settled, supernatant was removed and 1 mL WI solution (Appendix A) was added.

2.2.5. Turbofect Mediated Transfection of Mammalian Cells

Cells were grown in a 48-well plate. When the cells reached 60-80% confluency, media on the cells were removed completely prior to polyplex preparation and 500 µL fresh medium was added. 500 ng DNA was added into 1.5 mL tubes containing 50 µL DMEM. Polymer was pipetted into the tubes and immediately mixed by tapping. Mixture was incubated for 30 minutes to enable polyplex formation. 50 µl mixture was transferred into each well of the 48-well plate. Polyplexes were incubated with the cells overnight.

2.2.6. Imaging

2.2.6.1. Confocal Fluorescence Microscopy

Olympus Fluoview FV1000, Leica SP5 confocal laser scanning microscopes and Visitron spinning disk microscope were used for imaging. 488 nm excitation laser was used for GFP and 543 nm laser was used for Cy3 excitation. GFP emission was detected between 505-530 nm and Cy3 emission was detected between 570-670 nm. Composite images were prepared using ImageJ software (National Institutes of Health, USA, version 1.41).

2.2.6.2. Floid Cell Imaging Station

Invitrogen EVOS FLoid Cell Imaging Station (Thermo Fisher, USA) was used to check GFP expression in transfected HEK239 cells 24 hours after transfection experiments. Green light setting was used to capture GFP emission and white light setting was used to capture brightfield images.

2.2.7. Calculation of Delivery Efficiency

For the plant protoplast experiments delivery efficiency was calculated as follows:

$$Delivery\ Efficiency(\%) = \frac{Number\ of\ GFP(+)\ protoplasts}{Total\ number\ of\ protoplasts} \cdot 100\%$$

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Nucleic Acid Delivery to Plant Cells

3.1.1. Optimization of Plasmid Delivery Conditions

3.1.1.1. Polymer Concentration

Since pHP was never used for delivery of nucleic acids to plant protoplasts, the working concentration of the polymer for this purpose was unknown. Therefore, different dilutions of this polymer solution were prepared and tested to determine the polymer concentration that provides highest plasmid delivery efficiency (Fig. 3.1). Results suggested that 2 mg/mL concentration of pHP yielded the highest delivery efficiency among other tested concentrations. Thus, 2 ng polymer is required to deliver 1 μg of plasmid DNA.

3.1.1.2. Protoplast Washing Solution

Once the working concentration of the polymer was determined, different protoplast washing solutions were tested to assess their effects on plasmid delivery. Since this delivery method depends on Van der Walls interaction, charged molecules in the washing media can have a negative effect on the overall delivery efficiency. On the other hand, protoplasts are very fragile after isolation and the washing step is crucial for the viability of protoplasts. It was indicated that low pH of the protoplast medium allows protoplasts to recover from the isolation stress (Morocz et al., 1990), thus two different acidic media, namely R45 and ppN6M, were selected and tested as washing solutions.

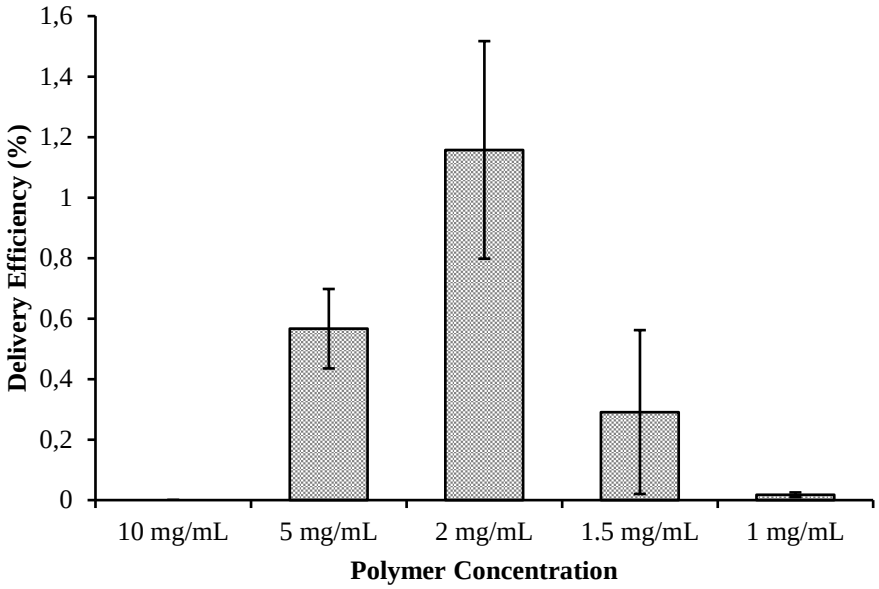

Figure 3.1. Optimization of polymer concentration

Maize protoplasts were treated with polyplexes formed with 1 µg GFP plasmid and indicated concentrations of cationic polymer. Delivery efficiencies were analyzed 24 hours after the polyplex treatment by counting the number of GFP expressing protoplasts under confocal laser scanning microscope. Graph shows mean values (± SD) from 3 independent experiments.

After the overnight protoplast isolation, protoplasts were washed with washing solutions to get rid of enzyme solution. Either ppN6M or R45 medium were used to wash protoplasts after overnight protoplast isolation (Figure 3.2). There were no significant difference between washing the protoplasts with R medium or ppN6M. We speculated that both media have their advantages and disadvantages. A rich medium such as ppN6M would be ideal to wash the protoplasts to preserve the viability but charged components may be inhibiting the cationic polymer or stabilizing the cell membrane. Alternatively, a simple medium like R medium would not interfere with the polyplex formation but viability will not be maintained with this medium either. Since delivery of the nucleic acids was more important, R medium was selected to wash the protoplasts in further experiments.

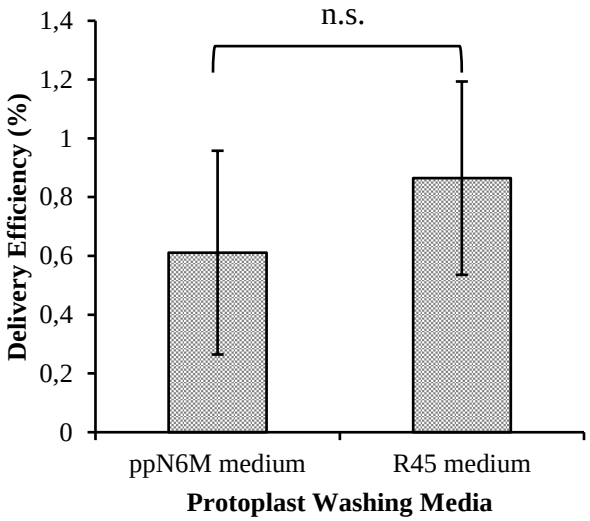

Figure 3.2. Optimization of protoplast washing solution

Maize protoplasts were washed with either ppN6M or R45 media after overnight protoplast isolation. Washed protoplasts were treated with polyplexes formed with 1 µg GFP plasmid and 1 µL of 2 mg/mL cationic polymer. Delivery efficiencies were analyzed 24 hours after the polyplex treatment by counting the number of GFP expressing protoplasts under confocal laser scanning microscope. Graph shows mean values (± SD) from 3 separate experiments. Student's t test (unpaired); n.s. not significant.

3.1.1.3. Incubation Time of Polyplexes with Protoplasts

Incubation of polyplexes with protoplasts is required for attachment of polyplexes to protoplast membranes. Different incubation durations were compared, however there was no significant difference between 5, 15, 30, 60 and 120 minutes (Fig. 3.3). It is important to note that even though 5-minute incubation could provide very high delivery efficiencies, there was a big variation between replicas. 15, 30 and 60-minute incubation times provided highest delivery efficiencies while having much less variance when compared to 5-minute incubation time. Therefore, protoplasts were incubated with the polyplexes for 30 minutes in further experiments.

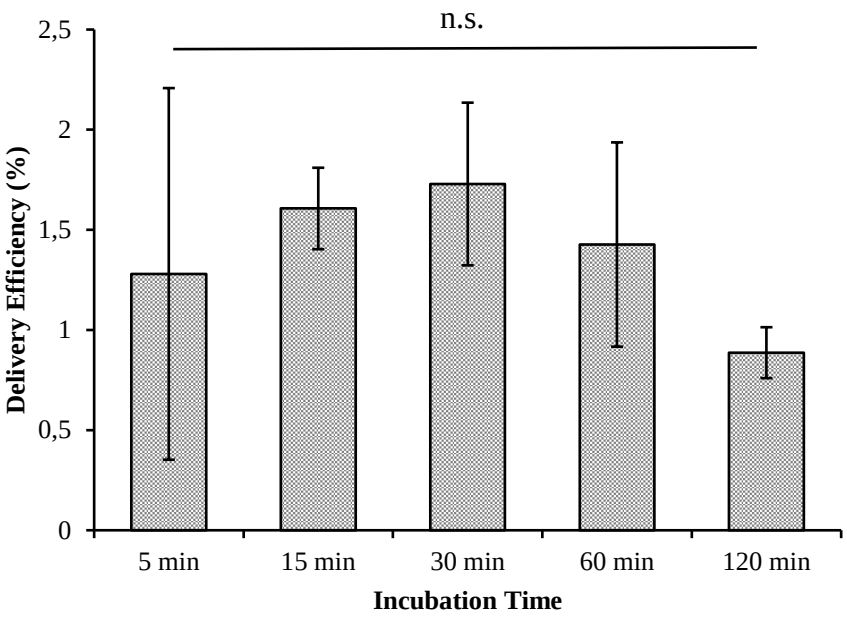

Figure 3.3. Optimization of protoplast incubation time with polyplexes

Maize protoplasts were incubated with polyplexes formed with 1 µg GFP plasmid and 1 µL of 2 mg/mL cationic polymer for indicated durations. At the end of these incubation periods, supernatants were removed and fresh protoplast media (ppN6M) were added. Delivery efficiencies were analyzed 24 hours after the polyplex treatment by counting the number of GFP expressing protoplasts under confocal laser scanning microscope. Graph shows mean values (± SD) from 3 independent experiments. One-way ANOVA test; n.s. not significant.

3.1.1.4. Plasmid to Polymer Ratio

Polyplexes should have a net positive charge in order to interact with the negatively charged cell membrane, so adjusting polymer to nucleic acid ratio is crucial. Cationic polymer can be neutralized by nucleic acids if insufficient amount of polymer is used. Several plasmid to polymer ratios were tested to determine the optimum ratio for highest delivery efficiency. Initially, keeping the polymer amount constant while increasing DNA concentrations were tested. Highest delivery efficiency was obtained with 1 µg DNA to 1 µL (2 mg/mL) polymer ratio (Fig. 3.4). It was shown that as the amount of DNA increased against constant amount of polymer, the delivery efficiency decreased. Since cationic polymer gets neutralized as it forms polyplexes with

negatively charged nucleic acids, increasing DNA amount against polymer could be preventing interaction of polyplexes with membranes.

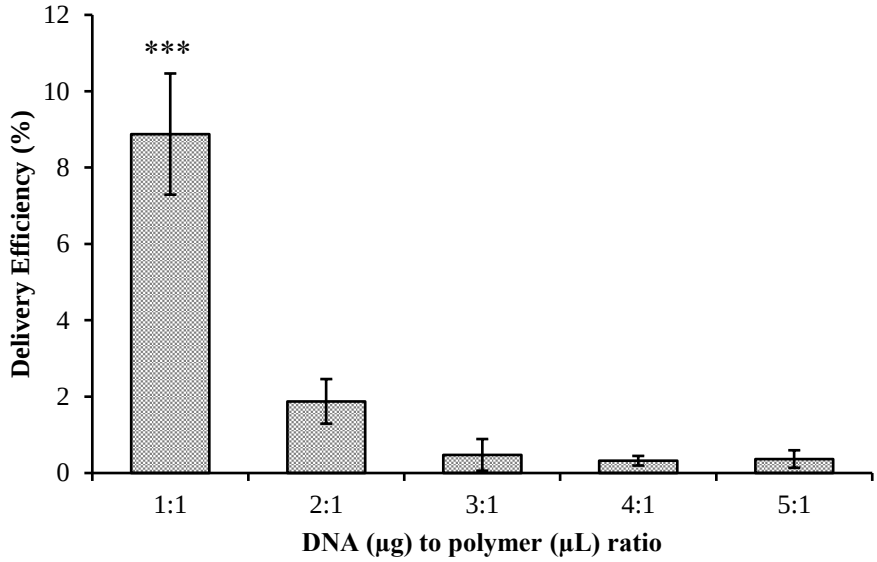

Figure 3.4. Optimization of plasmid to polymer ratios

Maize protoplasts were treated with polyplexes formed by indicated amounts of GFP plasmid and 1 µL of 2 mg/mL cationic polymer. Delivery efficiencies were analyzed 24 hours after the polyplex treatment by counting the number of GFP expressing protoplasts under confocal laser scanning microscope. Graph shows mean values (± SD) from 3 independent experiments. One way ANOVA test; ***P < 0.001.

3.1.1.5. Total Plasmid and Polymer Amounts

Increasing the DNA and polymer amounts while keeping the ratio constant was also tested. Previously optimized ratio was used while increasing the amounts of both DNA and polymer. Increasing DNA and polymer amounts adversely affected the delivery efficiency (Fig. 3.5).

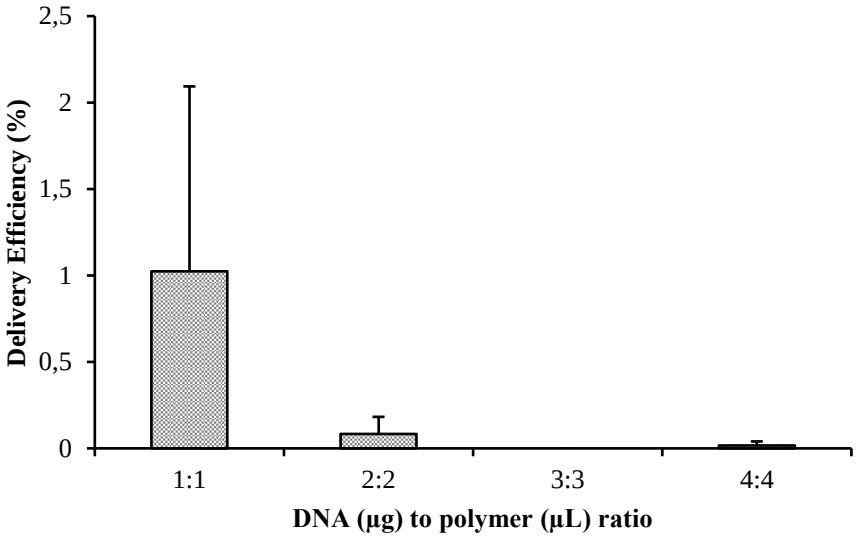

Figure 3.5. Optimization of plasmid and polymer amounts

Maize protoplasts were treated with polyplexes formed by indicated amounts of GFP plasmid and cationic polymer (2 mg/mL) in 1:1 ratio. Delivery efficiencies were analyzed 24 hours after the polyplex treatment by counting the number of GFP expressing protoplasts under confocal laser scanning microscope. Graph shows mean values (± SD) from 3 independent experiments.

3.1.1.6. Efficient Delivery of GFP Plasmid and Stable Transgenic Colony Formation

With the optimized conditions, up to 2000 GFP expressing protoplasts in 15,000 total protoplasts can be obtained by using 1 µg total DNA and 2 µg pHP in a volume of 200 µL (Fig. 3.6). This corresponds to up to 13% delivery efficiency in one well of an 8-well plate.

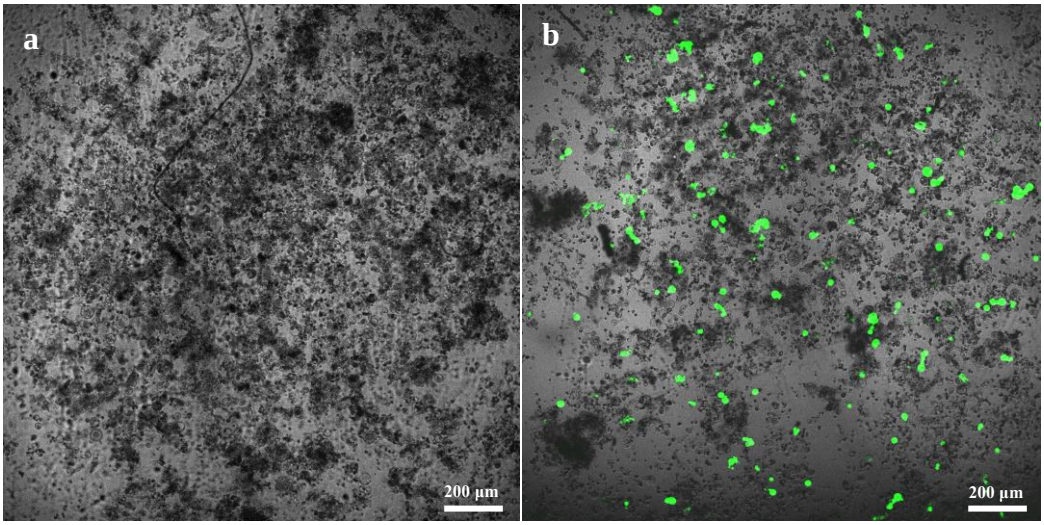

Figure 3.6. Efficient delivery of GFP plasmid

a. Untransformed control. b. 3 µL PCV of maize protoplasts were treated with polyplexes formed by 1 µg GFP plasmid and 1 µL of 2 mg/mL cationic polymer. 24 hours after the polyplex treatment, GFP expressing protoplasts were visualized under confocal laser scanning microscope Scale bars 200 µm.

Maintaining the mitotic activity of the transfected cells is as crucial as achieving high delivery efficiencies. Especially for OTNE, cell division/DNA replication is required for the nucleotide exchange. Therefore, the effect of cationic polymer based nucleic acid delivery on cell division activity was investigated. Cell divisions as soon as 1 day after polymer treatment could be observed, indicating that cell divisions were not adversely affected by the polymer treatment. Colony formation from a single GFP expressing protoplast could be achieved after nucleic acid delivery via the cationic polymer so that a new stable transgenic culture could be started (Fig. 3.7).

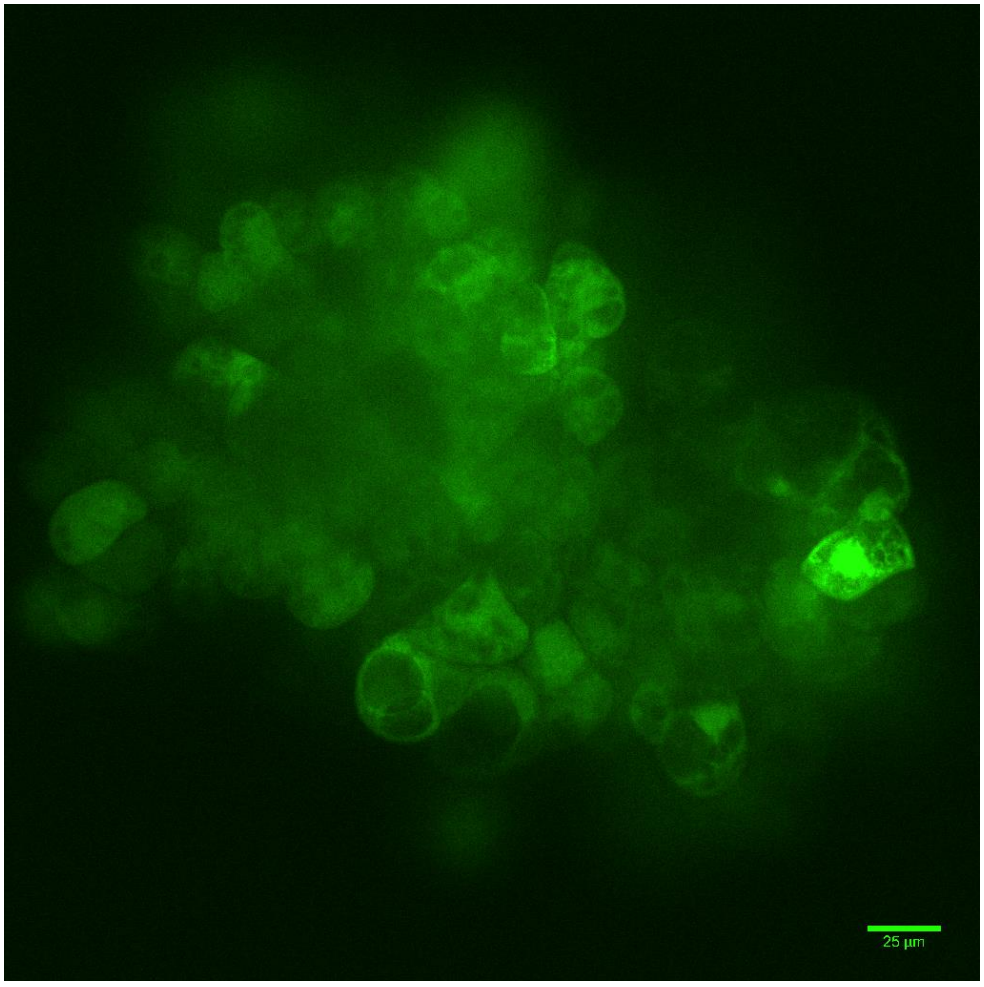

Figure 3.7. Stable transgenic GFP colony formation after GFP plasmid delivery with cationic polymer

Stable transgenic GFP colony formation 2 weeks after GFP plasmid delivery with cationic polymer. Scale bar 25 µm.

3.1.1.7. Plasmid Delivery to Different Plant Species

Applicability of this cationic polymer based nucleic acid delivery to other plant species was investigated. For this purpose, *Arabidopsis thaliana* Col. Gif. W. and *Oryza sativa* L. ssp. Japonica cv. 'Unggi 9' cultures were used. Same optimized protocol used for maize protoplasts was used for both plant species. 48 hours after treatment with polyplexes, protoplasts that expressed GFP were observed (Figure 3.8). It is noteworthy that number of transformed protoplasts were much lower when compared to maize experiments. However, it is possible to increase transformation

efficiency with further optimizations specific to each plant species. Nevertheless, pHP can be used for different plant species as well.

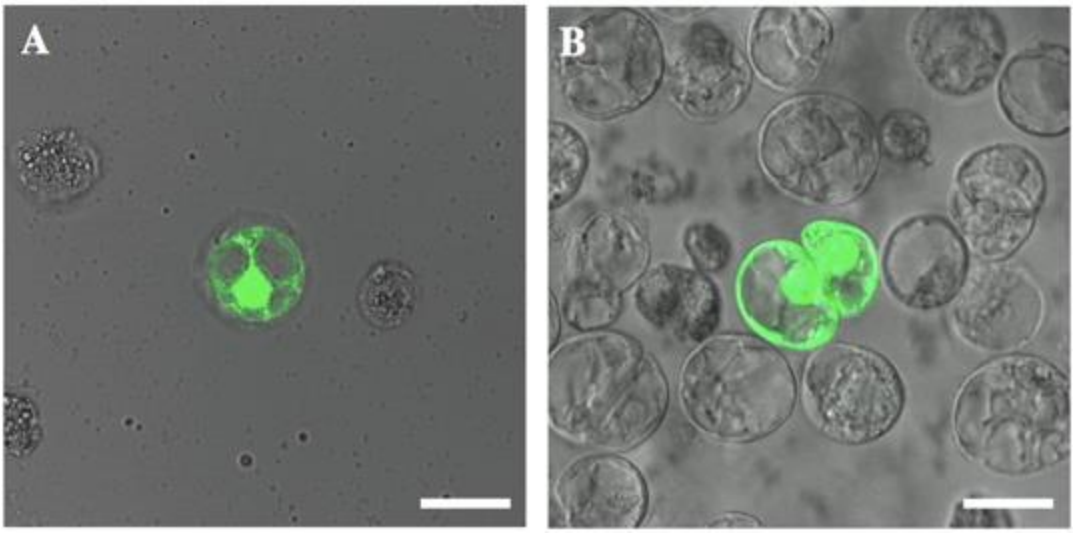

Figure 3.8. Plasmid delivery to rice and Arabidopsis protoplasts via pHP

3 µL PCV of either rice or Arabidopsis protoplasts were treated with polyplexes formed by 1 µg GFP plasmid and 1 µL of 2 mg/mL cationic polymer. 48 hours after the polyplex treatment, GFP expressing protoplasts were visualized under confocal laser scanning microscope. Scale bars 20 µm.

3.1.2. Optimization of Oligonucleotide Delivery

3.1.2.1. Oligonucleotide and Polymer Amounts

ssODN have different biochemical properties than plasmids. Consequently, optimized conditions for plasmid delivery may not be optimal for oligonucleotide delivery. Therefore, oligonucleotide delivery has to be optimized separately. Oligonucleotide delivery optimization is challenging since monitoring oligonucleotide delivery is difficult. For visualization purpose, fluorescently labeled ssODN were used and polyplex formation, attachment to protoplast membrane and nuclear accumulation were observed via confocal laser scanning microscopy.

Initially, only ssODNs or ssODNs that were incubated with pHP to form polyplexes were compared in their ability to attach cell membrane (Figure 3.9). It was clearly demonstrated that ssODNs alone cannot attach to the protoplast membranes, which

was determined by the absence of the ring structures around the protoplasts (Figure 3.9a). On the other hand, polyplexes were seen to attach to the membrane and form the ring formation that was visible under the microscope (Figure 3.9b). Additionally, it was observed that labeled ssODNs accumulated in protoplasts that had ruptured cell membranes.

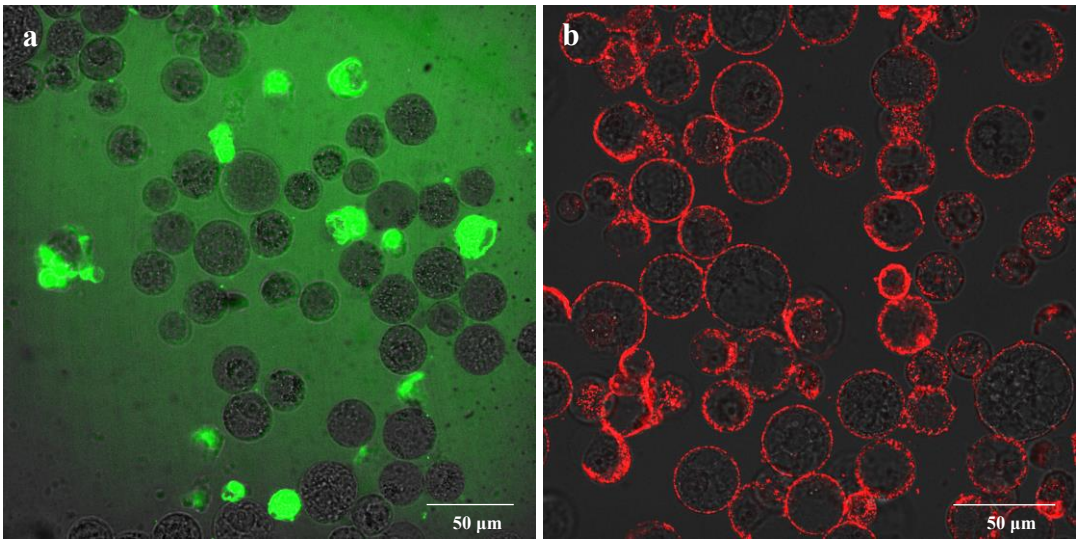

Figure 3.9. Interaction of ssODNs and ssODN polyplexes with protoplast membranes

a. 10 µM FAM-labeled ssODNs were incubated with maize protoplasts. 24 hours later, protoplasts were visualized under confocal laser scanning microscope. b. 3 µL PCV of maizee protoplasts were treated with polyplexes formed by 1 µg Cy3 labeled correction oligonucleotides and 1 µL of 2 mg/mL cationic polymer in 25 µL R75 medium. Protoplasts were visualized immediately after adding the polyplexes. Scale bars 50 µm.

Next, different ssODN amounts while using the optimized nucleic acid to cationic polymer ratio (1:1) were tested. 0.25, 0.5 and 1 µg Cy3 labeled ssODN were incubated with 0.25, 0.5 and 1 µL 2 mg/mL pHP respectively to form polyplexes. Immediately after adding the polyplexes onto protoplasts, the rings around the protoplasts were visualized to examine polyplex attachment to cell membrane (Figure 3.10a-c). To assess nuclear accumulation of labeled ssODNs, protoplasts were visualized 1 day after the treatment (Figure 3.10d-f). Since the oligonucleotides used in these

experiments were correction templates for OTNE mediated genome editing, cells were kept long-term and checked routinely for correction of mutant GFP.

In all conditions, polyplexes were able to attach to protoplast membranes and form red fluorescent rings around protoplasts (Figure 3.10a-c). This indicated that 1:1 ratio for ssODN (μg) and pHP (μL) allowed polyplexes to have a net positive charge and subsequently attach to negatively charged protoplast cell membrane. Although nuclear accumulation of Cy3 labeled ssODNs was observed 1 day after the treatment in all conditions, there were significant differences regarding the condition of the protoplasts (Figure 3.10d-f). Higher ssODN and pHP amounts correlated with decreased protoplast viability. Protoplasts treated with polyplexes formed by 1 μg ssODN and 1 μL pHP had the worst viability when compared with other treatments and as expected, majority of the protoplasts died by day 2. This can be explained due to adverse affects of excess polymer molecules. As a result, low oligonucleotide and pHP amounts such as 0.25 μg ssODN and 0.25 μL pHP can be used to achive sufficient nuclear accumulation while preserving culture viability.

3.1.2.2. Effect of Dilution

According to the previous observations, polyplex size increased proportionally to incubation time and polyplexes continued to attach to protoplast membranes until the whole surface area was covered with polyplexes. Additionally, it was possible to observe tiny polyplexes in the protoplast cytoplasm occasionally but bigger polyplexes, even though they were present abundantly in the environment, were not observed within the protoplasts.

Following these observations, it was hypothesized that as the polyplex size increases, transformation efficiency decreases. In order to test the hypothesis, polyplexes were prepared in diluted or undiluted conditions. Undiluted condition referred to addition of ssODNs and polymer into 25 μL of R75 medium. Then, the volume was completed to 200 μL and polyplexes were added onto protoplasts. In diluted condition, ssODNs and polymer were added directly to 200 μL of R75 medium and subsequently added onto protoplasts. Adding the ssODNs and polymer to a large volume was used to slow down the polyplex enlargement since in such environment, it takes more time for DNA

and polymer molecules to find each other and form complexes. Therefore, smaller complexes could be added onto the protoplasts in the diluted condition. In the undiluted condition, interaction of DNA and polymer molecules is more probable, thus this condition results in larger complexes.

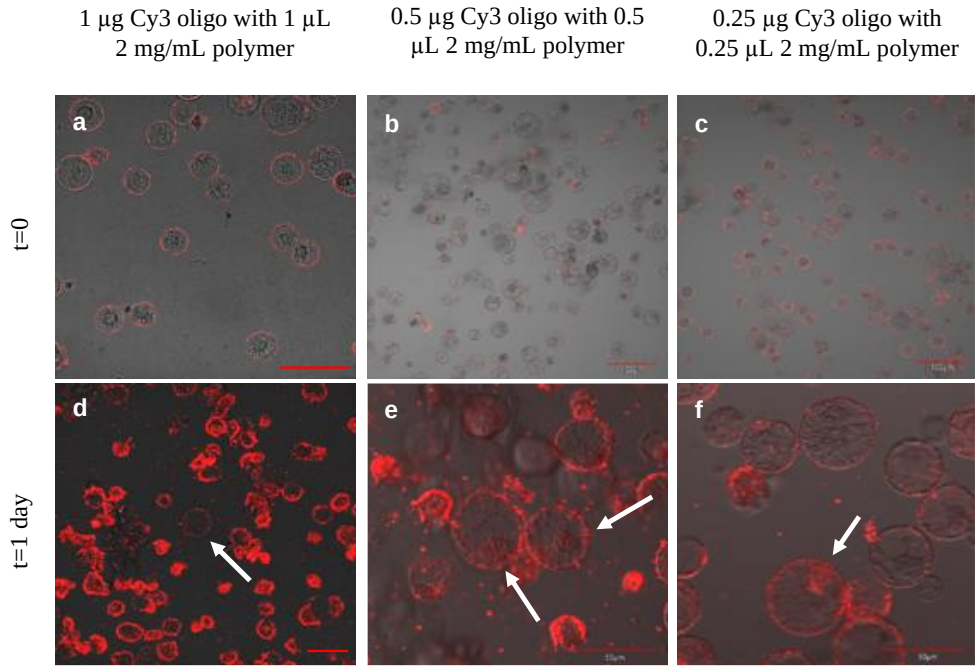

Figure 3.10. Comparison of oligonucleotide and polymer amounts

3 µL PCV of mazie protoplasts were treated with polyplexes formed by 0.25, 0.5 or 1 µg Cy3 labeled correction oligonucleotides and 0.25, 0.5 or 1 µL of 2 mg/mL cationic polymer respectively in 25 µL R75 medium. (a-c) Protoplasts were visualized immediately after adding the polyplexes. Scale bars 100 µm. (d-f) Protoplasts were visualized 24 hours after the polyplex treatment. Scale bars 50 µm.

Same ssODN and polymer amounts were tested in these experiments (Figure 3.10). Polyplexes were prepared in diluted or undiluted conditions for all ssODN/polymer amounts and incubated with the protoplasts for 1 day. Nuclear accumulation of the labeled ssODN indicated delivery efficiency.

Overall, it was clear that diluted condition resulted in less cell death when compared to undiluted condition for all ssODN/polymer amounts. Consistent with previous results, higher ssODN/polymer amounts correlated with more cell death in both diluted and undiluted conditions. Both 0.5 µg ssODN with 0.5 µL pHP and 0.25 µg

ssODN with 0.25 µL pHP in diluted condition can be used to achive sufficient nuclear accumulation while preserving culture viability.

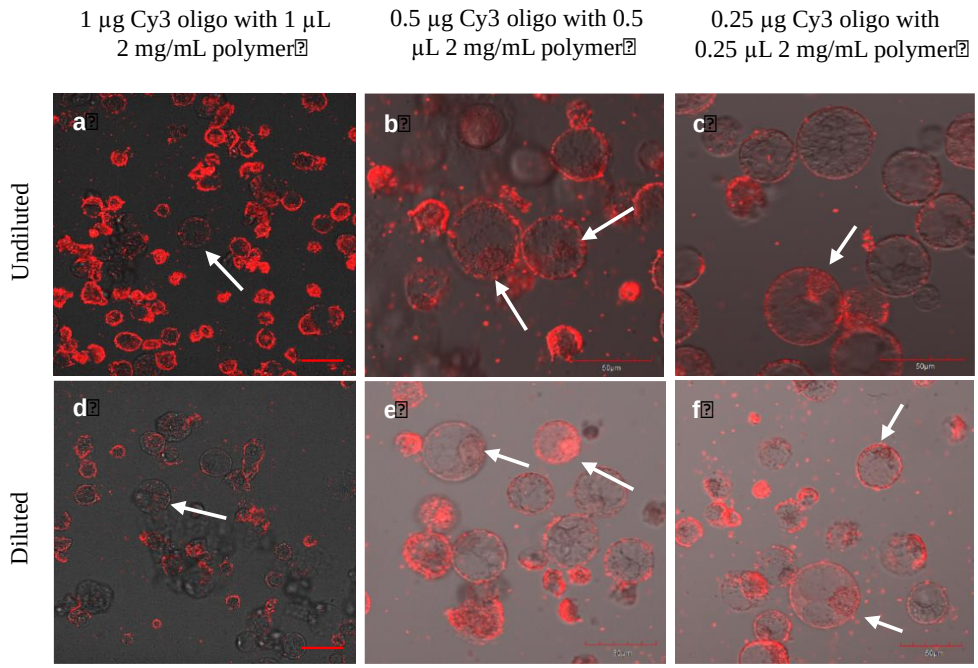

Figure 3.11. Comparison of nuclear accumulation of Cy3 labeled oligonucleotides in diluted or undiluted conditions

3 µL PCV of maize protoplasts were treated with polyplexes formed by 0.25, 0.5 or 1 µg Cy3 labeled correction oligonucleotides and 0.25, 0.5 or 1 µL of 2 mg/mL cationic polymer respectively in (a-c) 25 µL (undiluted) or (d-f) 200 µL (diluted) R75 medium. Protoplasts were visualized 24 hours after the polyplex treatment. Scale bars 50 µm.

3.2. Comparison of Plant Cell Transformation by pHP or PEG

PEG-induced direct DNA uptake is a widely used technique for introduction of nucleic acids to plant protoplasts. Although their mechanisms are different, polymer and PEG based nucleic acid delivery methods can be used for similar applications, such as genome editing via CRISPR/Cas9 system or OTNE.

3.2.1. Fusion Comparison

One of the disadvantages of PEG method is that plant regeneration after nucleic acid delivery is hindered due to protoplast fusions. Spontaneous nuclear fusions during mitosis have been observed in protoplasts that contain multi nuclei (Power et al., 1971). This can cause genomic instabilities and limit the mitotic activity of the culture. In order to check whether PEG-induced direct DNA uptake method causes enhanced protoplast fusions when compared to cationic polymer mediated nucleic acid delivery, PEG-induced DNA uptake method from Yoo et al. (2007) or the optimized cationic polymer based method was used to deliver GFP plasmid to maize protoplasts. Protoplast fusions were assessed after plasmid delivery with both methods by counting the nuclei of the protoplasts after DAPI staining (Fig. 3.12).

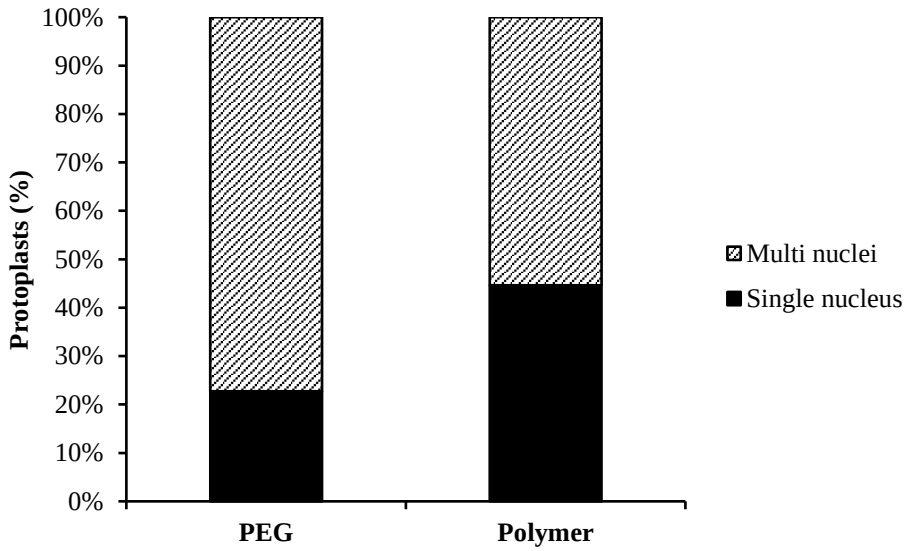

Figure 3.12. Protoplast fusions after plasmid delivery with PEG and polymer methods

10 µL PCV maize protoplasts were treated either with polyplexes or PEG. Polyplexes were formed with 10 µg of GFP plasmid and 10 µL of 2 mg/mL cationic polymer. 110 µL PEG was added onto 10 µg GFP plasmid and protoplast mixture. 24 hours after the treatments, protoplast nuclei were stained with nuclear dye DAPI and number of DAPI stained nuclei was counted under confocal laser scanning microscope for each treatment.

After PEG treatment, more than 75% of the protoplast population had multi nuclei. In polymer treated protoplasts, nearly half of the protoplast population had single nucleus

while the other half had multi nuclei. These findings show that PEG-induced direct DNA uptake method causes more protoplast fusions than cationic polymer mediated DNA delivery method.

3.2.2. Transformation Efficiency Comparison

As a following step, plasmid delivery efficiencies of PEG and polymer methods were compared. 10 µg plasmid was delivered to 10 µL PCV of maize protoplasts (Fig. 3.13). The results demonstrate that same amount of plasmid can be delivered to more protoplasts with cationic polymer method, indicating that under similar conditions, cationic polymer mediated DNA delivery is significantly more efficient than PEG-induced direct DNA uptake method.

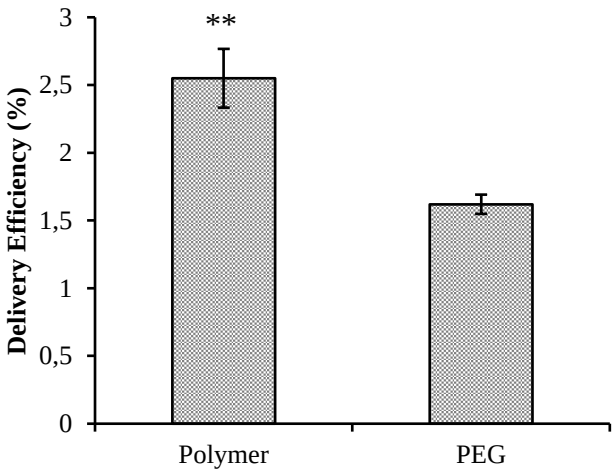

Figure 3.13. Comparison of large scale plasmid delivery efiiciencies of PEG and polymer methods

10 µL PCV maize protoplasts were treated either with polyplexes or PEG. Polyplexes were formed by 10 µg of GFP plasmid and 10 µL of 2 mg/mL cationic polymer. 110 µL PEG was added onto 10 µg GFP plasmid and protoplast mixture. Delivery efficiencies were analyzed 24 hours after the treatments by counting the number of GFP expressing protoplasts under confocal laser scanning microscope. Graph shows mean values (± SD) from 3 independent experiments. Student's t test (unpaired); **P < 0.05.

3.3. Nucleic Acid Delivery to Mammalian Cells via pHP

3.3.1. Optimization of Nucleic Acid Delivery Conditions

Zaliauskiene et al. previously described a transfection protocol for pHP and demonstrated efficient plasmid delivery in various mammalian cell lines including HeLa and HEK293 (2010). However, there can be variations in the final product due to the nature of the polymer synthesis process. Consequently, delivery protocol with the newly synthesized polymer had to be optimized.

In order to determine the working concentration of the in-house synthesized pHP for animal cell culture, transfection efficiencies using different polymer concentrations were compared. Lyophilized polymer was prepared at the final concentration of 10 mg/mL and this stock was referred as 'Undiluted Polymer'. Different dilutions of this stock were prepared by mixing the polymer with dH_2O.

Widely used commercial transfection reagent Turbofect was used along with different dilutions of pHP in order to compare the delivery efficiencies. Both pHP and Turbofect are cationic polymers, thus their mode of action is similar. Same amount of GFP expression plasmid (pSUPER.retro.neo+GFP) was delivered to HEK239 cells using either Turbofect or different dilutions of pHP. HEK293 cell line was used in these experiments since it is known to be easily transfected.

GFP expressing cell number indicated the delivery efficiencies of different treatments (Figure 3.14). Undiluted polymer (10 mg/mL) was observed to be more efficient than Turbofect. However, undiluted polymer also resulted in higher number of dead cells. We speculate that concentrated polymer solution allows higher delivery efficiency while decreasing the viability by disturbing the cell membrane beyond repair. It can be possible to preserve cell viability during cationic polymer mediated nucleic acid delivery by adding cell membrane stabilizing reagents to the reaction mix.

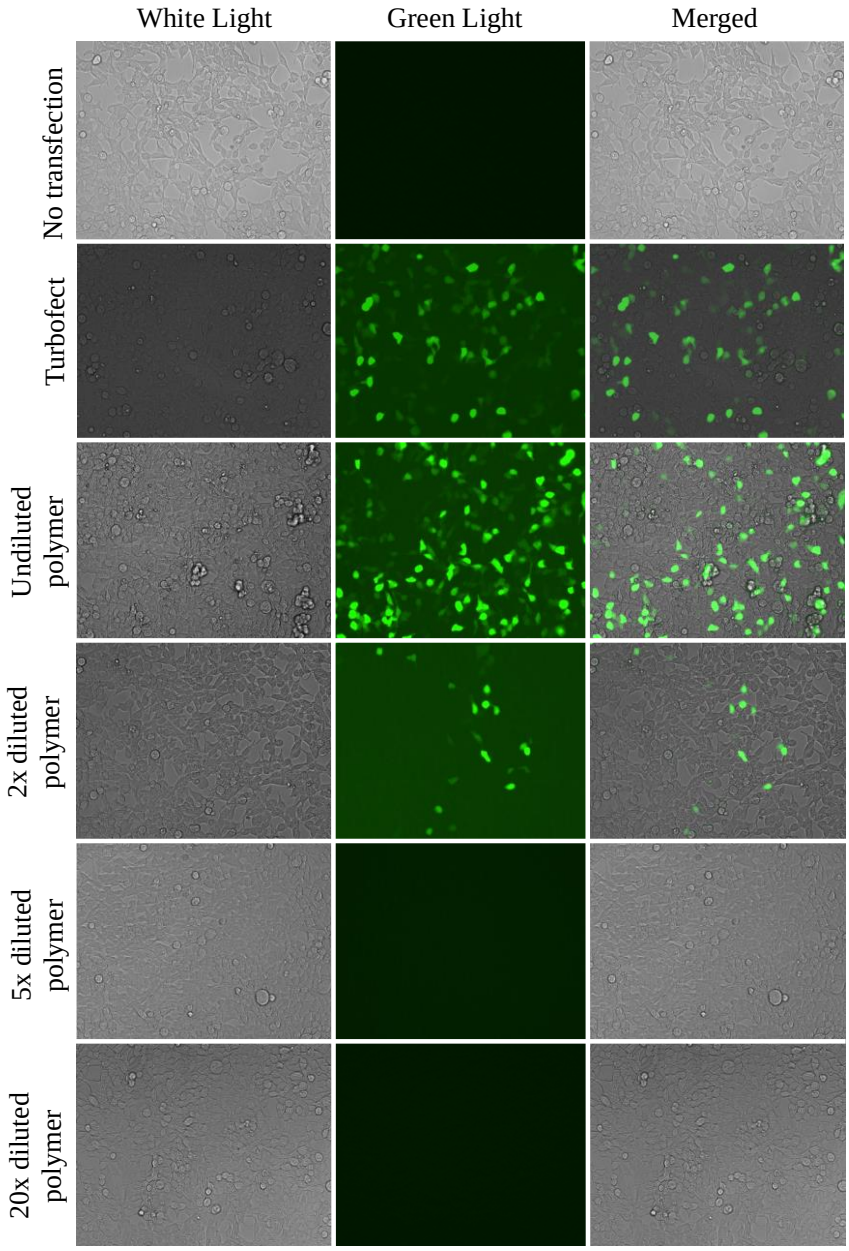

Figure 3.14. Optimization of polymer concentration

HEK293 cells were grown in a 48-well plate and when they reached 80% confluency, they were transfected with pScrambled using either turbofect or different dilutions of pHP. GFP expression was checked 48 hours after the transfection with Floid imaging station.

2 mg/mL pHP was less effective in plasmid delivery than 10 mg/mL for HEK293 cells. This highlights the differences between plant protoplasts and mammalian cells

since 10 mg/mL pHP was too potent for plant protoplasts, whereas for mammalian cells this concentration of pHP seems to be optimal. The underlying reason for such a difference can be due to the fact that plant protoplast cell membrane is much more fragile than animal cell membrane.

3.4. OTNE Mediated Genome Editing

For OTNE, delivering a ssODN to serve as a template for the endogenous repair machinery is sufficient for targeted mutagenesis. Therefore, correction template for the mutant GFP gene was designed as a 38-nucleotide long ssODN and this particular ssODN was shown to be successful in correcting mutant GFP in particle bombardment experiments. Both native and phosphorothioate (PTO)-modified versions were tested for OTNE experiments using the previously optimized conditions. PTO-modification prevents exonuclease mediated degradation of ssODN, however it is thought to interfere with targeted mutagenesis.

It was not possible to observe any correction in OTNE experiments even though ssODNs were observed to accumulate in nuclei of several protoplasts (Figure 3.11). This can be due to the rare occurrence of OTNE, combined with low transformation efficiency of ssODNs.

3.5. CRISPR/Cas9 Mediated Genome Editing in Plant Cells

3.5.1. Knock-in Experiments

Both genomic and transient knock-in experiments were conducted by delivering all the components of CRISPR/Cas9 system with pHP. For genomic knock-in experiments, stable transgenic maize cell line GFP7, which contains mutant GFP gene (with premature STOP codon) within its genome was used. 81-nucleotide long template ssODN, Cas9 plasmid and gRNA plasmid were combined in 3:1:1 ratio. This ratio was selected since it was known to work in particle bombardment experiments. The total nucleic acid amount was kept as 1 μg, so; 0.6 μg oligonucleotide template, 0.2 μg of each plasmid were used per experiment. This nucleic acid mixture was mixed with 1 μL (2 mg/mL) cationic polymer to form polyplexes. It was not possible to

observe correction of the mutant GFP in genomic knock-in experiments. This could have been due to accumulated mutations in the GFP7 culture over many passages. Therefore, transient correction was tested instead.

For transient knock-in experiments, along with Cas9 and gRNA plasmids a third plasmid containing the mutated GFP gene (pEGAD) was delivered into H1233 maize cell line. Template ssODN, Cas9 plasmid, pEGAD and gRNA plasmid were combined in 3:0.5:0.5:1 ratio and the total nucleic acid amount was kept as 1 µg. As a result, 0.6 µg oligonucleotide template, 0.1 µg of Cas9 plasmid, 0.1 µg of pEGAD and 0.2 µg of gRNA plasmid were used per experiment. This nucleic acid mixture was combined with 1 µL (2 mg/mL) cationic polymer. It was not possible to observe correction of the mutant GFP in transient knock-in experiments either. It was speculated that the reason for unsuccessful knock-in could be due to low probability of HDR pathway combined with the complexity of the experimental set-up required for delivering a heterogeneous mixture of nucleic acids.

3.5.2. Knockout Experiments

Instead of the low probability and complex knock-in approach, relatively straight forward knock-out approach was tested. For this purpose, stable transgenic GFP expressing A5 maize suspension cultures were used. A5 transgenic cell line was generated by *Agrobacterium* mediated gene delivery method. GFP gene copy number in A5 cell line is estimated to be around 1-2 copies as a characteristic of *Agrobacterium* gene transfer method. It is important to note that A5 culture consisted of homogenously GFP expressing clusters and there were no GFP-lacking dividing clusters visible. After protoplast isolation, the culture was visualized under confocal laser scanning microscope to confirm that the protoplasts were uniformly GFP positive (Figure 3.15).

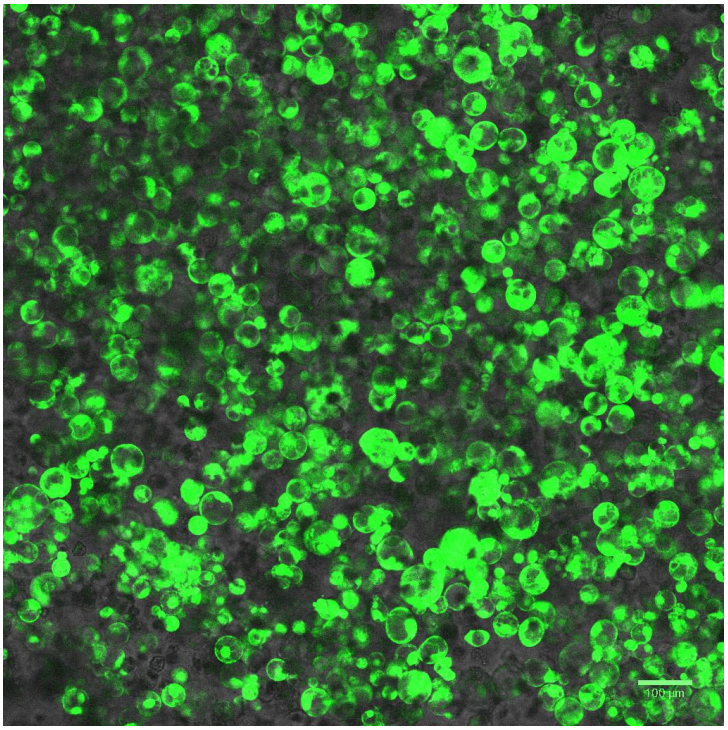

Figure 3.15. Uniformly GFP expessing protoplasts of A5 stable transgenic maize culture

Stable transgenic GFP expressing A5 maize suspension culture was used for CRISPR/Cas9 mediated knockout of GFP gene. Scale bar 100 µm.

Separate plasmids encoding gRNA and Cas9 were used for knockdown experiments and experiments were conducted in large scale. Total DNA amount was kept as 10 µg for 10 µL PCV and 1:1 gRNA plasmid to Cas9 plasmid ratio was used. Healthy and/or dividing cells without GFP signal were observed after polymer treatment. After 10 days, several actively dividing cell clusters lacking GFP signal were observed (Figure 3.16). This indicated that the knockdown was successful.

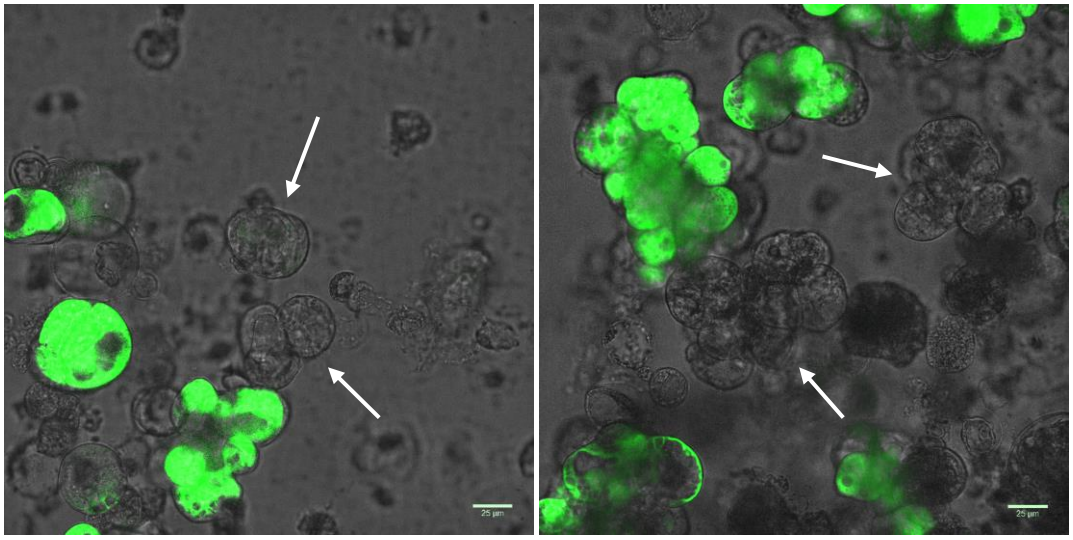

Figure 3.16. Cell clusters without GFP signal after GFP knockdown

Maize protoplasts were isolated from A5 cell culture and were treated with polyplexes formed by 0.5 µg Cas9 plasmid, 0.5 µg gRNA plasmid and 1 µL of 2 mg/mL cationic polymer. 11 days after the polyplex treatment cell clusters were visualized under confocal laser scanning microscope. Scale bar 25 µm.

CHAPTER 4

CONCLUSION

In this study, in-house synthetized cationic polymer pHP was optimized to yield significantly high delivery efficiency while preserving the viability of plant protoplasts. Polymer concentration, protoplast washing medium, DNA to polymer ratio and their amounts and incubation periods of polyplexes with the protoplasts were optimized for plasmid delivery. It was shown that the working concentration of the synthesized pHP was 2 mg/mL for maize protoplasts. DNA to polymer ratio optimization greatly enhanced protoplast transformation. It was shown that 1:1 ratio of DNA (μg) and polymer (μL) provided the highest plasmid delivery efficiency.

Separate optimization of oligonucleotide delivery was discovered to be necessary due to different properties of ssODNs and plasmids. One of the most significant finding was that if the polyplex sizes kept smaller, ssODN delivery to protoplasts were enhanced. Thus, nuclear accumulation of the fluorescently labeled ssODNs was more prominent when polyplexes were prepared in diluted condition. Furthermore, it was clearly demonstrated that when high amounts of polymer and ssODNs were introduced to the protoplasts, viability of the culture was adversely affected.

Applicability of pHP was demonstrated for transfection of mammalian cell lines. HEK239 cell line was used to compare transfection efficiency of pHP with routinely used transfection reagent Turbofect. 10 mg/mL pHP resulted in a higher transfection efficiency than Turbofect. However, the culture viability was also significantly less in 10 mg/mL pHP. It can be possible to decrease cell death caused by pHP if cell membrane protecting agents are also employed. However, this should be carefully investigated since it may adversely affect transfection efficiency. In future studies, ssODN delivery to mammalian cells can also be investigated using pHP.

Finally, CRISPR/Cas9 and OTNE based genome editing was investigated by delivering the editing components via pHP. It was not possible to demonstrate

CRISPR/Cas9 mediated knock-in and OTNE mediated gene correction, possibly due to their rare occurrence. Nevertheless, successful CRISPR/Cas9 mediated knockout of genomic GFP gene was demonstrated.

This study explores a new opportunity in plant transformation studies by demonstrating that a cationic polymer can be effectively used for plasmid and oligonucleotide delivery in plant protoplasts. Especially in an era, where genome editing technologies are getting more advanced by day, an established delivery system is necessary for these technologies to be successfully applied. Recently, CRISPR/Cas9 based genome editing was utilized to obtain cultivars with increased product yield, herbicide resistance and resistance to environmental stress conditions (Osakabe et al., 2016; Ghimire, 2017; Ledford, 2017). Advancements in plant transformation methods will surely encourage new research in the genome engineering field.

Future studies include synthesis of pHP derivatives to be investigated for their use in efficient nucleic acid delivery. These novel polymers can be compared with pHP and other transfection reagents in order to improve culture viability after polymer treatment as well as yielding higher nucleic acid delivery efficiency. Thus, commercialization of such polymers can be possible in the future to be used in plant cell transformation and transfection of mammalian cells.

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APPENDICES

A. BUFFERS AND SOLUTIONS

MAIZE SOLUTIONS

N6M Culture Media

Component	amount/L
KNO_3	2830 mg
$(NH_4)_2SO_4$	463 mg
$MgSO_4.7H_2O$	185 mg
$CaCl_2.2H_2O$	166 mg
KH_2PO_4	400 mg
Bacto-Tryptone	500 mg
Sucrose	30 g
Fe-Na-EDTA solution	5 mL
MS micronutrients	1 mL
N6 vitamin	1 mL
2,4-D (0.1 mg/ml)	5 mL

pH of the medium was adjusted to 5.8 with KOH.

ppN6-M/85 Solution

Component	amount/L
KNO_3	2830 mg
$(NH_4)_2SO_4$	463 mg
$MgSO_4.7H_2O$	370 mg
$CaCl_2.2H_2O$	300 mg
KH_2PO_4	400 mg

Bacto-Tryptone	500 mg
Sucrose	30 g
Glucose	80 g
Fe-Na-EDTA solution	5 mL
MS micronutrients	1 mL
N6 vitamin	1 mL
2,4-D (0.1mg/mL)	5 mL
NAA	1 mL
Zeatin	1 mL

pH of the medium was adjusted to 5.8 with HCl.

R Solution

Component	**amount/L**
$CaCl_2.2H_2O$	1.47 mg
$MgSO_4.7H_2O$	985 mg
KH_2PO_4	85 mg
MES-hydrate	600 mg

pH of the medium was adjusted to 5.7 with KOH.

For R45, 0.45 M D-mannitol was added to the final solution and for R75, 0.75 M D-mannitol was added to the final solution.

Plant Protoplasts Isolation Solution

Component	amount/10 mL
Enzyme Solution	1.2 mL
Protoplast Washing Solution	2 mL
sterile UP water	3.45 mL
BSA Solution (40 mg/mL)	0.5 mL
Antioxidant Solution (5 mg/mL)	0.1 mL
$CaCl_2.2H_2O$ (1M)	0.1 mL
$MgSO_4.7H_2O$ (1M)	0.1 mL
Osmotic Solution	2.5 mL

The solution was filter sterilized.

Enzyme Solution

Component	amount/10 mL
Cellulase RS	0.5 g
Pectolyase Y 23	0.025 g

Osmotic Solution

Component	amount/50mL
KNO_3	2.02 g
KH_2PO_4	1.38 g
K_2HPO_4	0.47 g
Glucose	4 g
Fructose	3.6 g
L-Proline	0.34 g

Protoplast Washing Solution

Component	amount/500mL
KNO_3	1414 mg
$MgSO_4.7H_2O$	92.5 mg
$CaCl_2.2H_2O$	83 mg
KH_2PO_4	200 mg
Bacto-Tryptone	250 mg
Sucrose	5 g
Glucose	27.5 g
Fructose	27.5 g
MS micronutrients	0.5 mg
N6 vitamin	0.5 mg

pH of the medium was adjusted to 5.8 with KOH.

Antioxidant Solution (5 mg/mL)

Component	amount/mL
n-propyl gallate	0.005 g
EtOH	0.2 mL
Ultrapure water	0.8 mL

ARABIDOPSIS SOLUTIONS

MM1 Culture Media

Component	amount/L
MS Salt Mix Basal	4.3 g
Sucrose	30 g
Vitamins (1000x)	1 mL

pH of the medium was adjusted to 5.6-5.8 with KOH.

Vitamins (1000x)

Component	amount/mL
myo-inositol	100 mg
Nicotinic acid	1 mg
Pyridoxine HCl	1 mg
Thiamine HCl	10 mg

RICE SOLUTIONS

G1 CULTURE MEDIUM

Component	amount/L
KNO_3	3 g
$NH_4H_2PO_4$	400 mg
$CaCl_2.2H_2O$	166 mg
$MgSO_4.7H_2O$	185 mg
Sucrose	30 g
G1 Micro (1000x)	1 mL
G1 Vitamins (1000x)	1 mL
Fe-Na-EDTA solution	5.74 mL
2,4-D (1 mg/mL)	2 mL

pH of the medium was adjusted to 5.5 with KOH.

G1 Micro (1000x)

Component	amount/10 mL
$MnSO_4.H_2O$	33 mg
$ZnSO_4.7H_2O$	15 mg
H_3BO_3	16 mg
KI	8 mg
$CuSO4.5H_2O$ (100x stock solution)	0.25 mg
$NaMoO4.2H_2O$	2,5 mg
$CoCl_2.6H_2O$ (100x stock solution)	0.25 mg

G1 Vitamins (1000x)

Component	amount/10 mL
Nicotinic acid	5 mg
Pyridoxine HCl	5 mg
Thiamine HCl	10 mg
Glycine	20 mg

PEG SOLUTIONS

WI Solution

Prepare 4 mM MES (pH 5.7) containing 0.5 M mannitol and 20 mM KCl.

W5 Solution

Prepare 2mM MES (pH 5.7) containing 154mM NaCl, 125mM $CaCl_2$ and 5 mM KCl.

MMG Solution

Prepare 4 mM MES (pH 5.7) containing 0.4 M mannitol and 15mM $MgCl_2$.

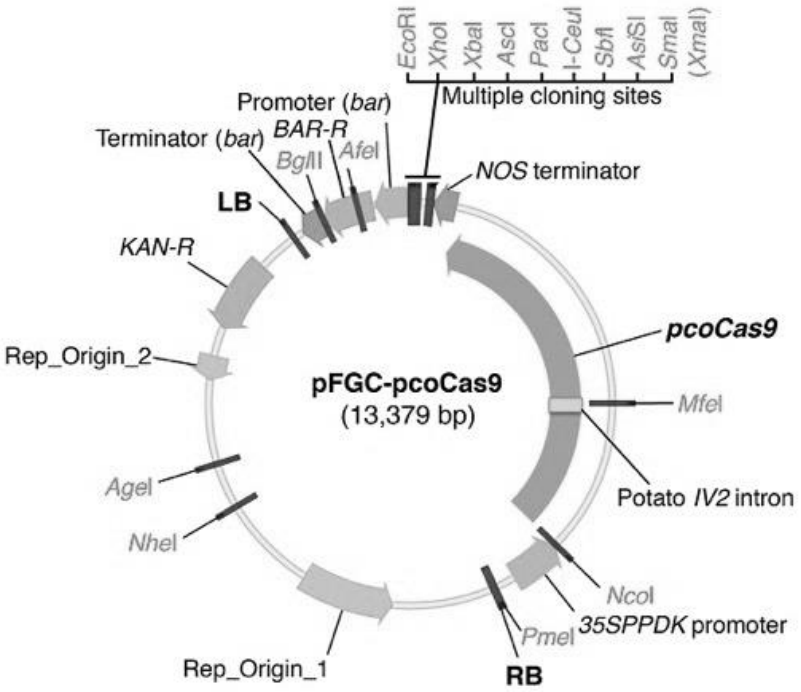
PEG-Calcium Solution

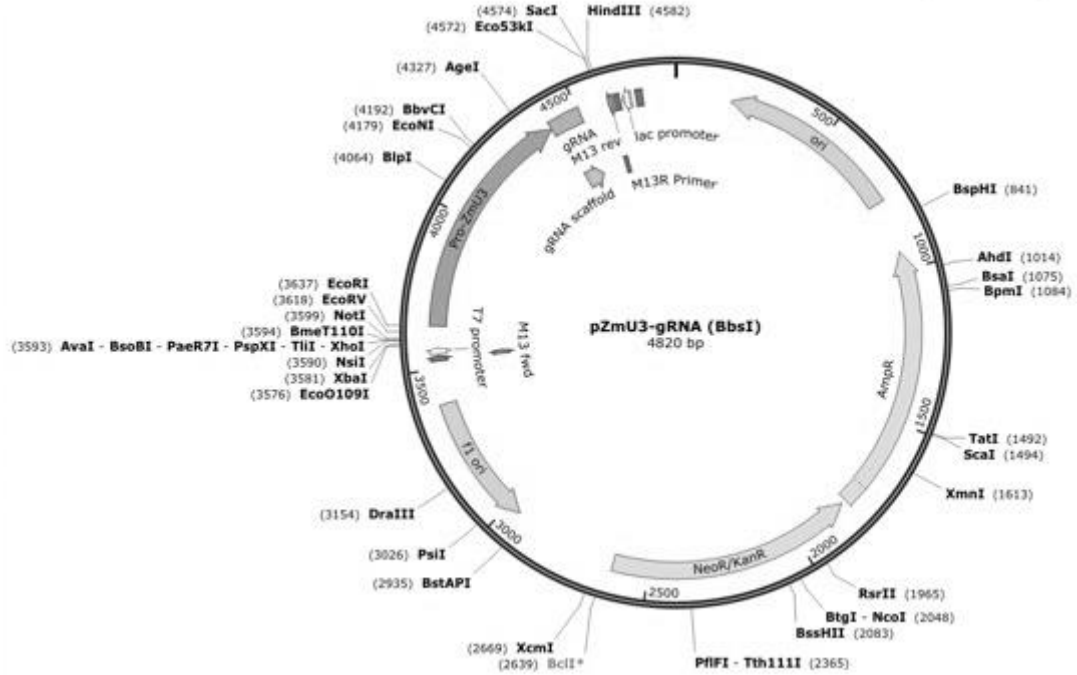
Prepare 20–40% (wt/vol) PEG4000 in ddH2O containing 0.2 M mannitol and 100 mM $CaCl_2$. It is important to prepare PEG solution at least 1 hour before transfection to completely dissolve PEG. The PEG solution should be used within 5 days.

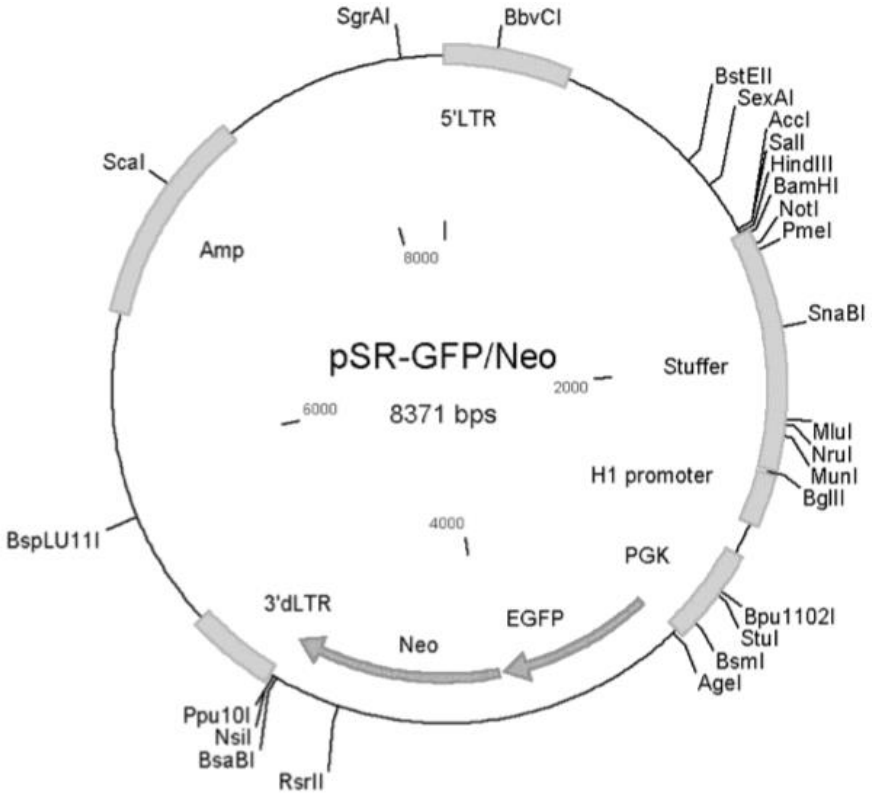
B. OLIGONUCLEOTIDE SEQUENCES

Name	Sequence (5'-3')	Length
mGFP correction ssODN	ATGGGCAAGGGCGAGGAGC TGTTCACTGGCGTGGTCCC	38 nt
CRISPR knock-in template ssODN	GATTGGGACCACGCCAGTG AACAGCTCCTCGCCCTTGCC CATGGACCGGGGATCCT CTAGAGTCGACCTGCAGA AGTAACA	81 nt

C. PLASMID MAPS






SgrAI
BbvCI
BstEII
SexAI
AccI
SalI
HindIII
BamHI
NotI
PmeI
SnaBI
MluI
NruI
MunI
BglII
Bpu1102I
StuI
BsmI
AgeI
ScaI
Amp
8000
5'LTR
pSR-GFP/Neo
8371 bps
2000
Stuffer
6000
H1 promoter
4000
PGK
EGFP
Neo
3'dLTR
BspLU11I
Ppu10I
NsiI
BsaBI
RsrII