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RESEARCH ARTICLE

Gene delivery into isolated *Arabidopsis thaliana* protoplasts and intact leaves using cationic, α-helical polypeptide

Nan Zheng[1,2*], Ziyuan Song[1*], Yang Liu[1], Lichen Yin (✉)[3], Jianjun Cheng (✉)[1]

1 Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, IL 61801, USA
2 State Key Laboratory of Fine Chemicals, Department of Polymer Science and Engineering, School of Chemical Engineering, Dalian University of Technology, Dalian 116024, China
3 Institute of Functional Nano & Soft Materials (FUNSOM), Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Collaborative Innovation Center of Suzhou Nano Science and Technology, Soochow University, Suzhou 215123, China

Abstract The application of gene delivery materials has been mainly focused on mammalian cells while rarely extended to plant engineering. Cationic polymers and lipids have been widely utilized to efficiently deliver DNA and siRNA into mammalian cells. However, their application in plant cells is limited due to the different membrane structures and the presence of plant cell walls. In this study, we developed the cationic, α-helical polypeptide that can effectively deliver DNA into both isolated *Arabidopsis thaliana* protoplasts and intact leaves. The PPABLG was able to condense DNA to form nanocomplexes, and they exhibited significantly improved transfection efficiencies compared with commercial transfection reagent Lipofectamine 2000 and classical cell penetrating peptides such as poly(L-lysine), HIV-TAT, arginine9, and poly(L-arginine). This study therefore widens the utilities of helical polypeptide as a unique category of gene delivery materials, and may find their promising applications toward plant gene delivery.

Keywords α-helical polypeptide, plant gene delivery, protoplast, intact leaves, transfection

1 Introduction

Plant gene delivery, which involves the introduction of exogenous genes into plant cells, has emerged as a promising tool for plant biotechnology. During the past decades, various transgenic methods have been developed for plant cells, such as Agrobacterium-mediated transformation [1], virus-mediated method [2], polymer and liposome-mediated method [3–5], bombardment biolistic-based method [6,7], poly(ethylene glycol) (PEG) transformation [8], and electroporation method [9]. However, most of these strategies suffer from serious translational limitations, including the low transfection efficiency, high cost, and host restrictions [10–12]. Therefore, the development of efficient gene delivery systems is of great significance in the plant biotechnology field.

Cell penetrating peptides (CPPs) have been utilized to deliver nucleic acids into plant cells [13], such as mung beans, soy beans [14], *Nicotiana benthamiana*, and *Arabidopsis thaliana* leaves [15], due to their excellent membrane penetration and translocation ability. For instance, histones have been demonstrated to be able to penetrate the plant cell membranes and also move through the cell walls [16,17]. Guanidinium-rich molecular transporters (GR-MoTrs) and oligoarginines (Arg9) have also been demonstrated to deliver small molecules, proteins, and DNA across the cell walls of microalgae [18,19]. It has also been revealed that the α-helical structure plays an essential role towards the membrane penetrating capabilities of CPPs. However, when used as gene delivery vectors in either mammalian cells or plant cells, CPPs often lack sufficient cationic charge density and backbone length, and thus are unable to effectively condense and deliver genes by themselves [20,21]. As such, CPPs often serve as membrane-penetrating materials incorporated or conjugated to existing delivery systems to achieve improved delivery efficiency [17,22–24] .

To address the critical challenges of CPP-mediated gene delivery, we developed the cationic, α-helical polypeptides, and demonstrated their unique potency toward non-viral gene delivery in mammalian cells [25,26]. Particularly, poly(γ-4-((2-(piperidin-1-yl)ethyl)aminomethyl)benzyl-L-glutamate) (PPABLG) was developed via a

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E-mails: lcyin@suda.edu.cn(Yin L), jianjunc@illinois.edu(Cheng J)

*These authors contributed equally to this work.

controlled ring-opening polymerization (ROP) method (Scheme 1(A)), and it possessed sufficient backbone length and simultaneously afforded stable helical secondary structure. As such, PPABLG has demonstrated its potency in condensing genes and delivering them into mammalian cells [27–30]. However, whether this potent membrane-penetrating material will work on plant genetic transformation is unknown. Similar to the gene delivery in mammalian cells, plant cell gene delivery is also hurdled by the various extracellular and intracellular barriers. The major difference between plant and mammalian cells is the cell walls surrounding the plant cell membranes [12,15,31–33]. Most of plant cell walls are composed of polysaccharides such as cellulose, hemicellulose and pectin, affording pores with the sizes ranging from 5 to 20 nm. Plant cell walls act as an obstacle that prevents most of the nanoparticles from entering the cells. Considering the excellent membrane activities of the helical polypeptides in mammalian cells, it is of great interest to further explore its utilities toward plant cell gene delivery. Therefore, we herein synthesized the helical polypeptide PPABLG, and first investigated the gene transfection efficiency of polypeptide/DNA complexes in *Arabidopsis thaliana* protoplasts isolated from *Arabidopsis thaliana* cells upon removal of cell walls. Since protoplast offered a similar cell membrane internalization mechanism to mammalian cells [34,35], it is commonly used as a model system to study plant cell physiological and cellular internalization. Because the direct delivery of genetic materials to intact plant tissues containing cell walls is much more essential for plant genetic engineering, we further evaluated the transfection efficiencies of PPABLG/DNA complexes in intact *Arabidopsis thaliana* leaves using the direct infiltration method, and compared to commercial transfection reagent as well as classical CPPs (Scheme 1(B)).

2 Experimental

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Anhydrous tetrahydrofuran (THF), hexane, and dimethylformamide (DMF) were dried by a column packed with 4Å molecular sieves and stored in a glovebox. Dry nitrobenzene (NB) was prepared by treating regular NB with CaH_2 followed by distillation under reduced pressure. Hexamethyldisilazane (HMDS) and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) were transferred to a glovebox and dissolved in anhydrous DMF. γ-(4-Vinylbenzyl)-L-glutamate *N*-carboxyanhydride (VB-L-Glu-NCA) was prepared as previously reported [25].

Arabidopsis thaliana plants were grown in a mixture of vermiculite, perlite, and peat moss at a 1 : 1 : 1 ratio in a temperature room under day-length conditions (16 h light and 8 h dark) at 22 °C. The plasmid DNA, pEGFP, encoding the super folder version of green fluorescent protein (GFP), was amplified in *E. coli* strain and extracted by a Plasmid Giga Kit. The plasmid DNA selected the cauliflower mosaic virus 35S as the promoter, which gives high level of expression in most plant cell types, including mesophyll protoplasts [36]. The *Arabidopsis thaliana* plant leaves and plasmid DNA were obtained from Ray Zielinski's lab (Urbana, IL, USA).

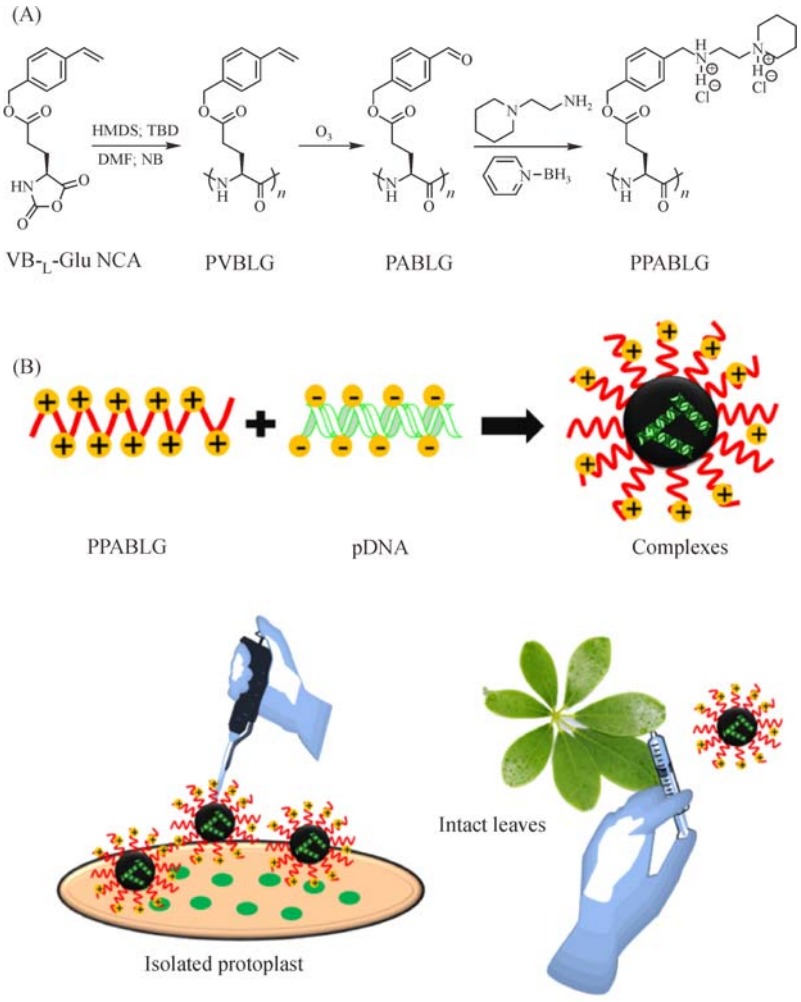
2.2 Instrumentation

Circular dichroism (CD) measurements were carried out on a JASCO J-815 CD spectrometer (JASCO, Easton, MD, USA). Polypeptide was dissolved in deionized water at a concentration of 0.2 mg/mL at pH 7.0, and was placed in a quartz cell with a pathlength of 0.1 cm. The mean residue molar ellipticity of the helical polypeptide was calculated based on the measured apparent ellipticity following the reported formula [26]:

$$\text{Ellipticity } ([\theta],\ \text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}) = \frac{\text{Millidegree} \times \text{Mean residue weight}}{\text{Pathlength(mm)} \times \text{Concentration(mg}\cdot\text{mL}^{-1})}$$

2.3 Synthesis of PPABLG

PPABLG was synthesized according to the reported procedure [25]. Briefly, VB-L-Glu-NCA (58 mg, 0.2 mmol) was dissolved in DMF (0.9 mL) in a glove box, followed by addition of nitrobenzene (30 μL), HMDS (20.0 μL, 0.1 mol/L, M/I = 100), and TBD solution (20.0 μL, 0.01 mol/L) in DMF. Fourier transform infrared spectroscopy was used to monitor the polymerization until the conversion reached 99% (within 48 h at room temperature (RT)) to obtain the poly(γ-4-vinylbenzyl-L-glutamate) (PVBLG). Methanol (100 μL), benzyl chloroformate (50 μL), and *N*,*N*-diisopropylethylamine (DIEA, 50 μL) were added and stirred for 3 h to cap the amino end groups. DMF was removed under vacuum, and the resulting polymer was precipitated using hexane/ether (1 : 1, v/v) and collected by centrifugation at 4000 r/min. PVBLG was then dissolved in chloroform (30 mL) and oxidized by O_3 at −78 °C. Dimethyl sulfide (1 mL) was added and the solution was stirred at RT overnight before the solvent was removed under vacuum. The product poly(γ-4-aldehydebenzyl-L-glutamate) (PABLG) was precipitated using methanol (45 × 3 mL) to remove impurities, and collected by centrifugation. The resulting PABLG (35 mg) was dissolved in DMF (2 mL), where 1-(2-aminoethyl)piperidine (162 μL, 10 molar equivalents relative to the Glu repeating unit) was added and the solution was stirred at 50 °C for 24 h. It then reacted with borane pyridine as the reducing agent (141 μL, 10 molar



Scheme 1 (A) Synthetic routes of PPABLG; (B) schematic illustration of the formation of PPABLG/DNA complexes and gene delivery in both isolated protoplasts and intact leaves

equivalents relative to the Glu repeating unit) at 50 °C for another 24 h. HCl (5 mol/L, 1 mL) was added to quench the boranes and protonate the amine groups, and the final product PPABLG was dialyzed against deionized water (MWCO = 1 kDa) and lyophilized.

2.4 Protoplast isolation

Arabidopsis thaliana leaves (10 pieces, 2 cm × 5 cm, w × d) were harvested from 3−5 week old plants and were pasted upside down to standard lab tape which has been stuck to the lab bench. A strip of Magic tape (3 mol/L, St. Paul, MN, USA) was then applied to the underside of each leaf. The Magic tape was then carefully removed from the lab tape to peel off the lower epidermal surface cell layer. The peeled leaves were transferred to a petri dish containing 20 mL of enzyme solution (1% cellulase R-10, 0.2% macerozyme R-10, 0.4 mol/L mannitol, 10 mmol/L $CaCl_2$, 20 mmol/L KCl, 0.1% BSA and 20 mmol/L MES, pH 5.7). The mixture was then incubated at 20 °C with gentle shaking for 60−90 min until the protoplasts were released into the solution. The digested mixture was filtered through a 70-μm filter into a round-bottom centrifuge tube. The obtaining protoplasts were washed by W5 solution (154 mmol/L NaCl, 125 mmol/L $CaCl_2$, 5 mmol/L KCl, 2 mmol/L MES, pH 5.7) and kept on ice for 30 min to allow them to recover from the stress of isolation. The protoplasts were then collected by centrifugation at 100 × g for 1−2 min and re-suspended in MMg solution (0.4 mol/L mannitol, 15 mmol/L $MgCl_2$, 4 mmol/L MES, pH 5.7) to a final concentration of 1−2 × 10^5 cells/mL.

2.5 Preparation and characterization of PPABLG/pDNA complexes

pEGFP which encodes a super folder version of EGFP was provided by Ray Zielinski's lab and used as reporter gene

[37]. PPABLG and pDNA were dissolved in deionized water at 0.2 mg/mL. To form complexes, PPABLG was added into the pDNA solution at various N/P ratios (2, 5, 10, 20, 30, 40, and 50) followed by vortex for 30 s and incubation at RT for 20 min. Gel retardation assay was used to evaluate the DNA condensation by loading the freshly prepared complexes on a 1% agarose gel at 100 ng DNA/well followed by electrophoresis at 100 V for 45 min. The migration of pDNA was observed by the gel documentation. The EB exclusion assay was further performed to quantify the DNA condensation level. DNA was stained with EB at the DNA/EB weight ratio of 10 and RT for 1 h. PPABLG was then added into the DNA/EB solution at various N/P ratios followed by further incubation at RT for 30 min before quantification of the fluorescence intensity (λ_{ex} = 510 nm, λ_{em} = 590 nm). The DNA condensation efficiency (%) was calculated according to the following equation:

$$\text{DNA condensation efficiency}(\%) = \left(1-\frac{F-F_{EB}}{F_0-F_{EB}}\right)\times 100$$

where F_{EB}, F, and F_0 denote the fluorescence intensity of pure EB solution, DNA/EB solution with PPABLG, and DNA/EB solution, respectively. Particle size and zeta potential of freshly prepared complexes were immediately evaluated or evaluated after different incubation time (20, 40, 60, 90 and 120 min) by dynamic laser scanning on a Malvern Zetasizer (Herrenberg, Germany).

2.6 Protoplast transfection assay

Arabidopsis thaliana protoplasts were diluted with the W5 solution to a concentration of 1×10^5 cells/mL in a 6-well plate, into which PPABLG/DNA complexes at various N/P ratios (10, 20, 30 and 40) were added at 0.5, 1, 2 and 5 μg DNA/well. Lipofectamine 2000 (LPF) was used according to the manufacturer's protocol. Poly(L-lysine)/DNA (PLL/DNA), HIV-TAT/DNA (TAT/DNA), arginine9/DNA (Arg9/DNA), and poly(L-arginine)/DNA (PLR/DNA) complex were also evaluated as controls. The N/P ratios of control groups were determined by the optimal results based on literatures [38,39]. After incubation at RT for 12 h, W5 solution (3 mL) was added slowly and the protoplasts were pelleted by centrifugation at 100 × g for 1 min and re-suspended in W5 solution (1 mL) in a 6-well plate coated with 1% BSA at RT for 16 h in light. The EGFP expression was assessed by flow cytometry, and the transfection efficiency was expressed as percentage of EGFP-positive cells.

2.7 Transfections into intact leaf cells

PPABLG/DNA complexes containing 2 μg DNA (50 μL) were infiltrated directly into fully expanded *Arabidopsis thaliana* leaves using a syringe without a needle. The treated *Arabidopsis thaliana* plants were incubated under day-length conditions (16 h light/8 h dark) at 22 °C in a plant incubating room for up to three days. The treated leaves were cut into 1 cm^2 around the infiltrated section. Qualitative observation of EGFP expression in the leaves was performed by confocal laser scanning microscopy (LSM710, Zeiss, Germany). LPF, PLL, TAT, Arg9, and PLR at the N/P ratios of 5, 8, 15, 15 and 10 served as controls, respectively.

2.8 Statistical Analysis

Statistical differences in transfection efficiency were determined by Student's *t*-test with a two-tailed distribution, and differences were judged to be statistically significant at $^*p < 0.05$ and very significant at $^{**}p < 0.01$.

3 Results and discussion

3.1 Characterization of PPABLG and PPABLG/DNA complexes

PPABLG was synthesized via ROP of VB-L-Glu-NCA, and the subsequent ozonolysis and reductive amination of side-chains as previously reported [25]. The obtained PPABLG adopted typical α-helical structure as evidenced by the characteristic double minima at 208 and 222 nm in the CD spectrum (Fig. 1(A)). Because of the cationic nature of the helical polypeptide, PPABLG was able to condense the anionic DNA at the N/P ratio higher than 5 as evidenced by the gel retardation assay (Fig. 1(B)) and EB exclusion assay (Fig. 1(C)). At the N/P ratio $\geqslant$ 5, complexes with hydrodynamic diameters around 160 nm and positive zeta potential around + 36 mV were obtained (Fig. 1(D)). SEM image further revealed the spherical morphology of the complexes at the N/P ratio of 30 (Fig. 1(E)). Upon incubation for up to 2 h, the particle size of the complexes remained almost unaltered (slight increase to around 200 nm), indicating their desired stability (Fig. 1(F)).

3.2 Transfection into *Arabidopsis thaliana* protoplasts

The gene delivery efficiency of the helical polypeptides was first monitored in *Arabidopsis thaliana* protoplasts, which are deprived of the cell walls and commonly utilized as the model system for plant cell gene delivery studies. Transient GFP expression could be observed 28 h after incubation of the isolated protoplasts with complexes as determined by flow cytometry. Maximal transfection efficiency was noted when the N/P ratio was increased to 30, while a further increase led to notably decreased GFP

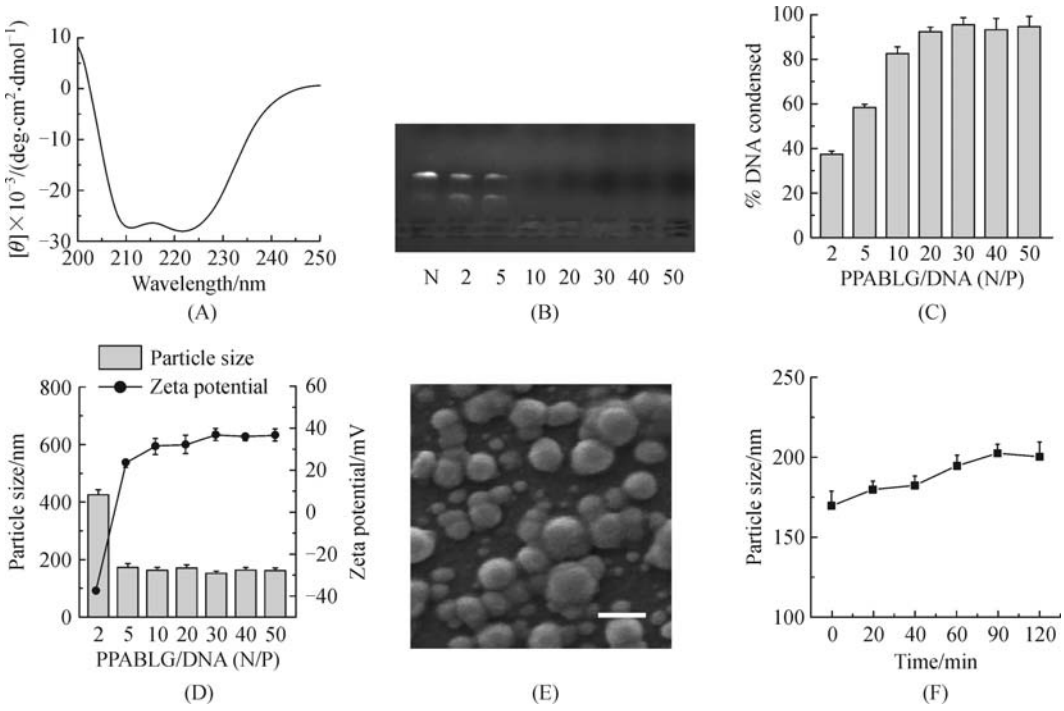


Fig. 1 (A) CD spectrum of PPABLG in deionized water (0.2 mg/mL) at pH 7.0; (B) DNA condensation by PPABLG at different N/P ratios as evaluated by the gel retardation assay. N represents naked DNA; (C) EB exclusion assay showing the condensation of DNA in the PPABLG/DNA complexes; (D) particle size and zeta potential of PPABLG/DNA complexes at various N/P ratios; (E) SEM image of PPABLG/DNA complexes at the N/P ratio of 30 (bar = 200 nm); (F) stability of PPABLG/DNA complexes (N/P = 30) following various incubation time at 37 °C

expression level, which could be attributed to the material toxicity at excessively high concentrations (Fig. 2(A)). Such observation was consistent with our previous findings that excessive membrane activity could cause irreversible cell damage [28]. An increase in the transfection efficiency was also noted when the DNA amount was increased from 0.5 to 5 μg per 10^5 cells (Fig. 2(A)). In direct comparison to the commercial transfection reagent LPF and other CPP-based transfection reagent including PLL, TAT, Arg9 and PLR, PPABLG also showed remarkably higher transfection efficiencies. While the transfection efficiency of 16% was lower than previously reported PEG-mediated protoplast transfection method (~40%)[1], the DNA amount used in this study was nearly 100-fold lower. These findings thus indicate that the helical polypeptide might be utilized as a promising gene delivery vector into plant protoplasts (Fig. 2(B)).

3.3 Transfection into intact *Arabidopsis thaliana* leaves

Plant cell walls are fibrous structures composed of cellulose, pectin, hemicellulose, lignin, and proteins, which serve as a notorious barrier for the delivery of external materials. To explore whether the helical polypeptide could penetrate the cell walls to achieve effective transfection in the intact leaf cells of *Arabidopsis thaliana*, the PPABLG/DNA complexes at various N/P ratios were directly infiltrated into the back side of the leaves without protoplast preparations. As shown in Fig. 3, the expression of GFP was clearly observed in the cytosol of the spongy mesophyll cells by CLSM (Figs. 3(C–E), which was obviously different from the autofluorescence of the chloroplasts (Fig. 3(A)). Generally, plant cell walls have pore sizes ranging from 5 to 30 nm, which prevent most of the nanoparticles with large particle size from transporting across the cell walls [31,40,41]. Helical polypeptides have been demonstrated as excellent membrane penetrating materials that can generate pores on mammalian cell membranes [30,42]. Therefore, the possible mechanism for the helical polypeptide–mediated gene delivery might be that the complexes penetrated into the cells by generating pores or enlarging the original pores on the cell walls. As shown in Figs. 3(C−E), complexes with N/P ratios of 20 and 30 both exhibited notable GFP transfection, while complexes with N/P ratio of 40 exhibited reduced GFP expression level, which was consistent with previous

1) Sheen J. A transient expression assay using Arabidopsis mesophyll protoplasts, 2002

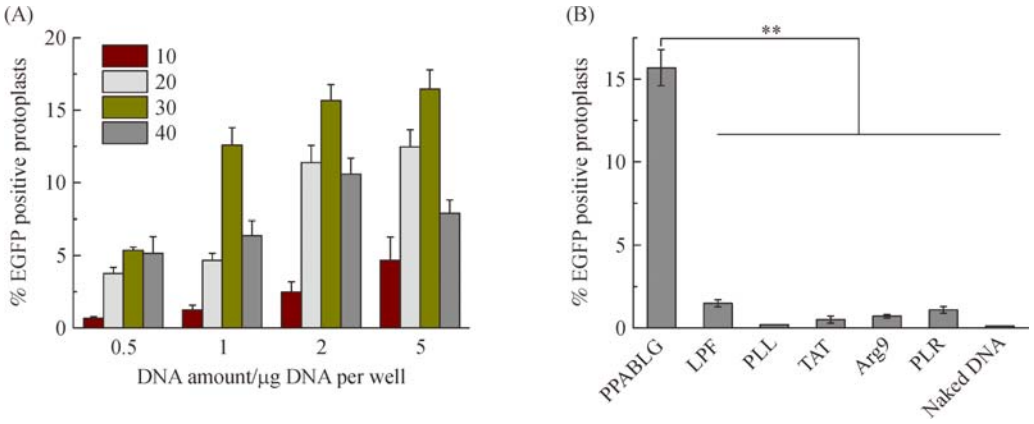


Fig. 2 (A) Transfection efficiencies of PPABLG/DNA complexes in *Arabidopsis thaliana* protoplast cells at various DNA amount and PPABLG/DNA N/P ratios ($n = 3$); (B) transfection efficiencies of various complexes in *Arabidopsis thaliana* protoplast cells ($n = 3$). DNA amount was maintained constant at 2 μg/10^5 cells. N represents naked DNA. LPF, PLL, TAT, Arg9, and PLR at the N/P ratios of 5, 8, 15, 15 and 10 served as controls, respectively

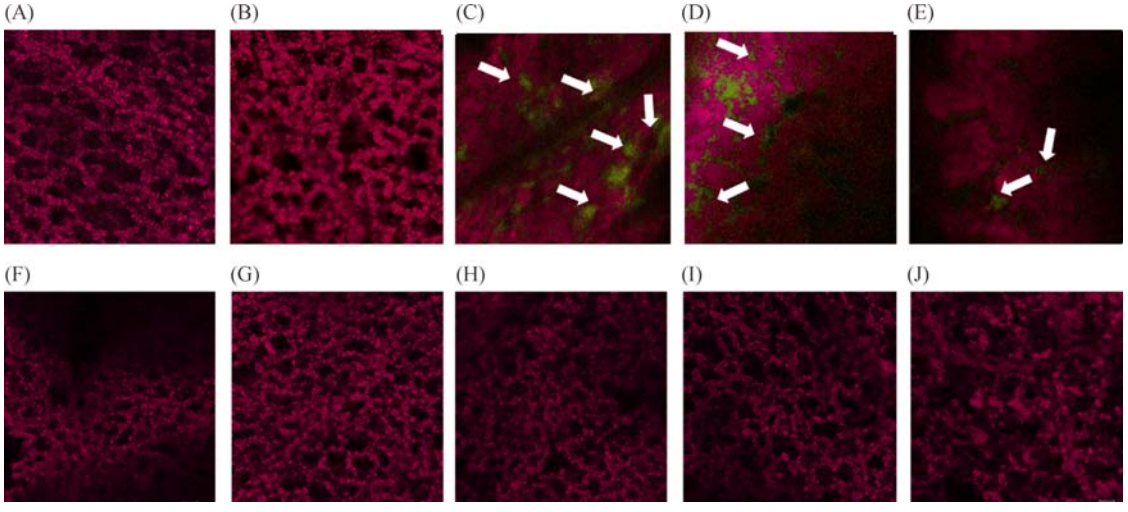


Fig. 3 Transfection efficiencies of polypeptide/DNA complexes in intact leaves as observed by CLSM. Spongy mesophyll cells of *Arabidopsis thaliana* leaves infiltrated with (A) ultrapure water; (B) naked DNA; (C) PPABLG/DNA complexes (N/P = 20); (D) PPABLG/DNA complexes (N/P = 30); (E) PPABLG/DNA complexes (N/P = 40); (F) LPF/DNA complexes (N/P = 5); (G) PLL/DNA complexes (N/P = 8); (H) TAT/DNA complexes (N/P = 15); (I) Arg9/DNA complexes (N/P = 15); (J) PLR/DNA complexes (N/P = 10). C, D, and E showed GFP expression (green, white arrows) in the cytosol, which was notably different from the autofluorescence of chloroplasts (red)

findings on the protoplast transfection. In comparison with commercial reagent and CPPs including LPF, PLL, TAT, Arg9, and PLR, significantly higher GFP expression levels were also noted for the helical PPABLG. These findings demonstrated that the cationic, helical polypeptide was capable of penetrating the plant cell walls to mediate effective gene transfection in intact leaves, and thus may serve as a promising gene delivery system for plant cell transfection.

4 Conclusions

In this study, we evaluated the potentials of cationic, α-helical polypeptide, a unique category of gene delivery material with potent membrane penetration capabilities, in mediating gene delivery in plant cells. We demonstrated that the helical polypeptide was able to mediate effective gene transfection in both isolated protoplasts and intact leaves, which indicated its capability to penetrate not only

cell membranes but also cells walls. These findings thus extended the application scope of helical polypeptide-mediated gene delivery, and may serve as a promising tool for plant cell genetic engineering.

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