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A gene delivery system for insect cells mediated by arginine-rich cell-penetrating peptides

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ABSTRACT

Most bioactive macromolecules, such as protein, DNA and RNA, basically cannot permeate into cells freely from outside the plasma membrane. Cell-penetrating peptides (CPPs) are a group of short peptides that possess the ability to traverse the cell membrane and have been considered as candidates for mediating gene and drug delivery into living cells. In this study, we demonstrate that three arginine-rich CPPs (SR9, HR9 and PR9) are able to form stable complexes with plasmid DNA and deliver DNA into insect Sf9 cells in a noncovalent manner. The transferred plasmid DNA containing enhanced green fluorescent protein (*EGFP*) and red fluorescent protein (*RFP*) coding regions could be expressed in cells functionally assayed at both the protein and RNA levels. Furthermore, treatment of cells with CPPs and CPP/DNA complexes resulted in a viability of 84–93% indicating these CPPs are not cytotoxic. These results suggest that arginine-rich CPPs appear to be a promising tool for insect transgenesis.

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1. Introduction

Insect cells have been widely used in recombinant baculovirus expression systems and transient gene expression studies (Ogay et al., 2006). The transfection of foreign DNA into insect cells is the first step for obtaining transient or stable expression of desired proteins. In recent years, a variety of transgenic tools have been developed in response to the need to generate transgenic insects for use in pest control (Lukacsovich et al., 2008; O'Brochta and Handler, 2008). In general, both viral and nonviral vector-mediated methods are two traditional DNA delivery systems (Luo and Saltzman, 2000). Especially, nonviral DNA delivery has been a prominent route over the past 40 years due to its ease of assembly and low levels of toxicity, immunogenicity and insertional mutagenesis. For example, microinjection, hydrodynamic force, ultrasonic nebulization, particle bombardment and electroporation are mechanical and electrical transfection techniques, while calcium phosphate, artificial lipids, peptides/proteins, dendrimers and others are chemical methods (Luo and Saltzman, 2000). In an attempt to establish an efficient and safe tool for nonviral DNA delivery, specific peptides were used as carriers to deliver DNA into insect cells in the present study.

Most macromolecules including DNA, RNA and protein are unable to traverse the cell membrane. Over two decades ago, two groups found that the Tat (transactivator of transcription) protein of the human immunodeficiency virus type 1 (HIV-1) possesses the ability to rapidly translocate through the plasma membrane and accumulate in the cell (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Eleven amino acids (YGRKKRRQRRR) in the middle region of the Tat protein were proven to be the key for the cellular uptake (Vives et al., 1997). Accordingly, short peptide sequences mainly composed of basic amino acids were named cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) (Wadia and Dowdy, 2002; Deshayes et al., 2010). CPPs possess the ability to not only penetrate the cell membrane, but also to deliver bioactive macromolecules, such as proteins, nucleic acids, peptide nucleic acids, inorganic particles and liposomes, into cells of various species (Wadia and Dowdy, 2002). This phenomenon in some cases was called protein transduction (Wadia and Dowdy, 2002). The cellular uptake of polyarginines, a few kinds of CPPs, tends to be more efficient than those of polylysine, polyhistidine or polyornithine (Futaki, 2002). Thus, CPPs represent an attractive approach in opening new perspectives for gene/drug delivery during the last decade (Deshayes et al., 2010).

Abbreviations: CecB, cecropin B; CPP, cell-penetrating peptide; Cy3, Cyanine 3; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; HIV-1, human immunodeficiency virus type 1; HR9, histidine-rich nona-arginine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; N/P, nitrogen (NH_3^+)/phosphate (PO_4^-); PBS, phosphate buffered saline; PEI, polyethylenimine; PR9, Pas nona-arginine; RFP, red fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction; SR9, synthetic nona-arginine; Tat, transactivator of transcription.

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Arginine-rich CPPs have been demonstrated in our laboratory to be able to transport fluorescent protein cargos, such as green fluorescent protein (GFP) and red fluorescent protein (RFP), into living animal and plant cells (Wang et al., 2006; Chang et al., 2007; Hou et al., 2007; Hu et al., 2009; Lu et al., 2010) and into other organisms including cyanobacteria, bacteria, archaea and yeasts (Liu et al., 2008) in a noncovalent manner. Moreover, we found that arginine-rich CPPs could deliver DNA (Chen et al., 2007; Dai et al., 2011; Lee et al., 2011), RNA (Wang et al., 2007) or nanoparticles (Liu et al., 2010a, 2010b, 2011; Xu et al., 2010) into live cells noncovalently. Accordingly, three arginine-rich CPPs, synthetic nona-arginine (SR9; R9), histidine-rich nona-arginine (HR9; C-5H-R9-5H-C) and Pas nona-arginine (PR9; FFLIPKG-R9), were investigated in our group (Dai et al., 2011; Liu et al., 2011). Applications of CPPs have recently drawn enormous attention as a powerful tool for the direct intracellular delivery in gene and protein therapeutics (Deshayes et al., 2010). However, limited study of CPPs (Palm et al., 2006; Cermenati et al., 2011; Chen et al., 2011) was presented in the field of Arthropoda which is the most numerous phylum of all living organisms on earth, both in number of species and in number of individuals.

In the present study, the major aims were to 1) investigate intracellular gene delivery mediated by three arginine-rich CPPs (SR9, HR9 and PR9) into insect cells at the protein level, 2) confirm the CPP-mediated gene delivery at the RNA level and compare the relative transfection efficiency of CPPs that form complexes with plasmid DNA in a noncovalent fashion and 3) determine the cytotoxicity of CPPs. To achieve these goals, we first analyzed the noncovalent interactions between CPPs and plasmid DNA. Plasmid DNA was *in vitro* labeled and transported by CPPs into Sf9 cells monitored by a fluorescent microscope. We then examined the functional assay of the CPP-mediated gene delivery using live cell imaging, flow cytometry and RT-PCR assay. To evaluate any cytotoxicity caused by CPPs, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay was finally conducted to determine cell viability of Sf9.

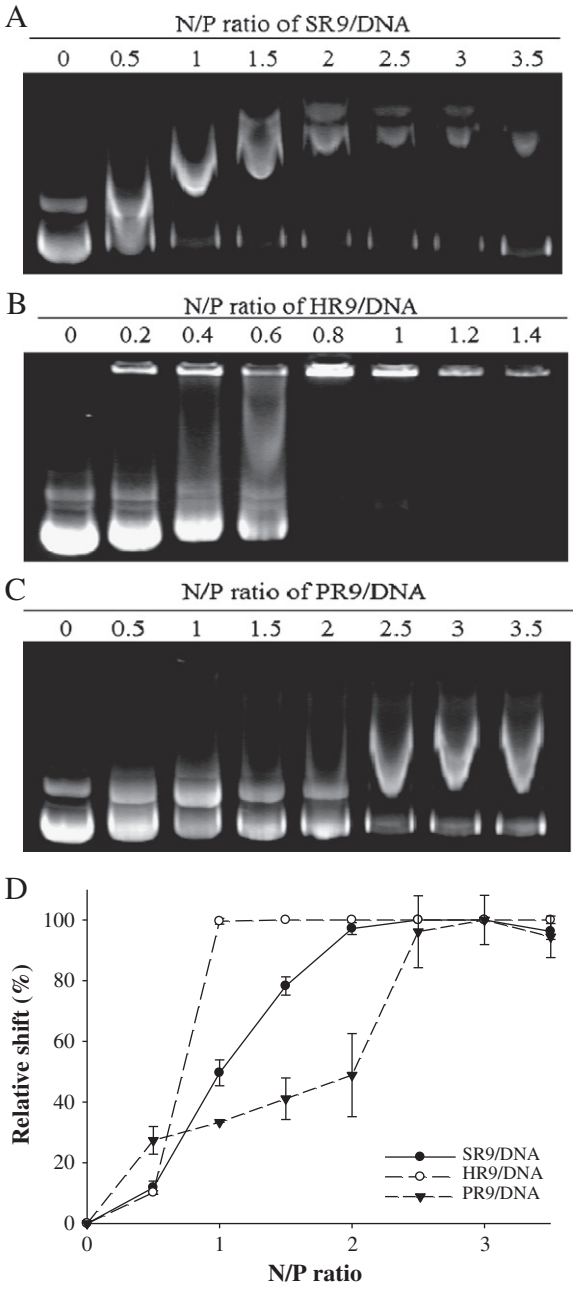


Fig. 1. Gel retardation assay of the interaction between arginine-rich CPP and plasmid DNA. The pBacCecBEGFP plasmid was mixed with SR9 at molar nitrogen/phosphate (NH_3^+/PO_4^- or N/P) ratios of 0 (DNA only), 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 (A), HR9 at N/P ratios of 0 (DNA only), 0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 (B) or PR9 at N/P ratios of 0 (DNA only), 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 (C). The mixtures were analyzed by electrophoresis on a 0.5% agarose gel and stained by EtdBr. Histogram of the relative shift of CPP/DNA complexes at different ratios (D). Data are presented as mean ± standard deviations from 3, 3 and 6 independent experiments for SR9, HR9 and PR9, respectively.

2. Materials and methods

2.1. Insect cells

Sf9 (*Spodoptera frugiperda*) insect cells (Bioresource Collection and Research Center, Hsinchu, Taiwan; BCRC 60011) were grown in the Grace's insect medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum. Cells were seeded at a density of 1×10^5 in each well of 24-well plates and incubated in an incubator at 28 °C as previously described (Chao et al., 1992).

2.2. Peptide preparation

Three arginine-rich CPPs (SR9, HR9 and PR9) were synthesized from Genomics Co. (Taipei, Taiwan) as previously described (Liu et al., 2011).

2.3. Gel retardation assay

In order to reveal the interaction between DNA and CPP, gel retardation was performed as previously described (Liu et al., 2011). Briefly, three μg of the pBacCecBEGFP plasmid (19.4 nM) were mixed with different amounts of SR9 or PR9 peptide (0, 16.7, 33.5, 50, 66.9, 83.6, 100 and 117 μM) to form various molar nitrogen/phosphate (NH_3^+/PO_4^- or N/P) ratios (0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5) or HR9 peptide to form various N/P ratios (0, 0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4) in phosphate buffered saline (PBS) at a final volume of 30 μl and incubated for 2 h at 37 °C. These complexes were analyzed by electrophoresis on a 0.5% agarose gel at 50 V for 40 min and stained by EtdBr.

2.4. Plasmid DNA labeling

The pBlueScript-SK + plasmid DNA (Agilent Technologies, Santa Clara, CA, USA) was *in vitro* labeled with the *Label*IT Cyanine 3 (Cy3) nucleic acid labeling kit according to the manufacturer's instructions (Mirus Bio, Madison, WI, USA) as previously described (Chen et al., 2007). SYBR Green I nucleic acid gel stain was used according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Luminescent images were captured using the Typhoon FLA 9000 biomolecular imager (GE Healthcare, Piscataway, NJ, USA) with the excitation wavelength at 473 nm of LD laser for SYBR Green I and with the excitation wavelength at 532 nm for Cy3 as previously described (Liu et al., 2011).

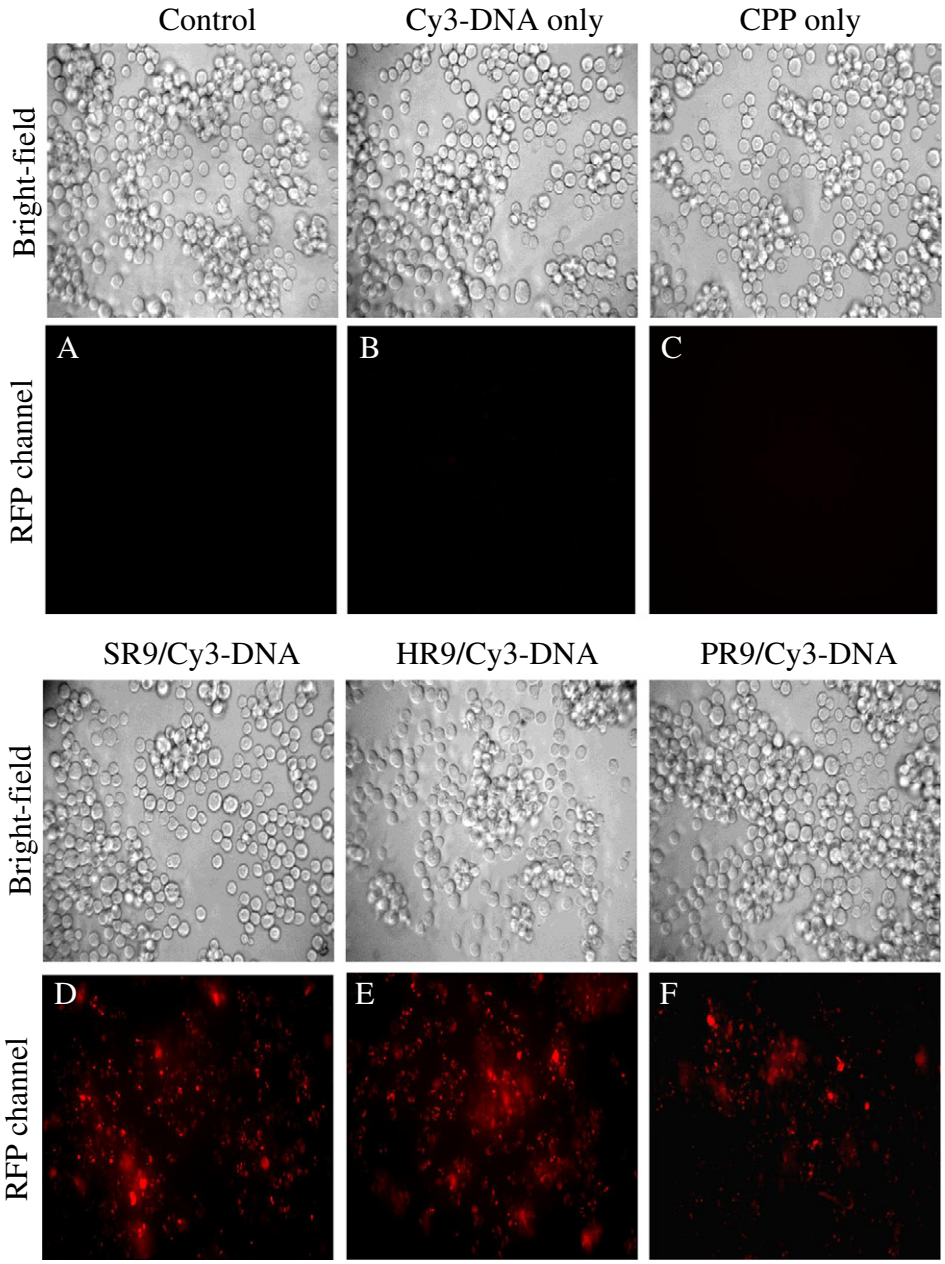


Fig. 2. Fluorescent microscopy of the CPP-mediated delivery of the Cy3-labeled DNA into Sf9 cells. Cells were treated with PBS as a control (A), the Cy3-labeled DNA only (B), SR9 only (C), SR9/Cy3-labeled DNA (D), HR9/Cy3-labeled DNA (E) or PR9/Cy3-labeled DNA (F) complexes. Images of bright-field and the RFP channel are shown from a confocal microscope at a magnification of 200×.

2.5. Plasmid construction

The pBacCecBEGFP-3XP3DsRed2 plasmid (kindly provided by Dr. Kiyoko Taniai, National Institute of Agrobiological Science, Japan) was described previously (Imamura et al., 2006). The RFP cassette containing the 3XP3 promoter, coding region of *DsRed2* and SV40 polyadenylation signal of the pBacCecBEGFP-3XP3DsRed2 plasmid was removed by digestion with *Bgl*II restriction enzyme, gel-purification and self-ligation to generate the pBacCecBEGFP plasmid. The resulting pBacCecBEGFP plasmid consists of the enhanced green fluorescent protein (*EGFP*) cassette including the strong *cecropin B* (*CecB*) gene promoter, coding region of *EGFP* and *3′CecB* with 7778 bp in total length.

2.6. CPP-mediated DNA delivery

Three μg of the Cy3-labeled pBlueScript-SK + plasmid DNA were mixed with CPPs (SR9, HR9 or PR9) at the N/P ratio of 3 in PBS at a final volume of 80 μl and incubated for 2 h at room temperature as previously described (Chen et al., 2007). The CPP/Cy3-labeled DNA complexes were added into each well of Sf9 cells in 24-well plates and incubated for 30 min at 28 °C. Cells were then washed thrice with PBS to remove CPP/Cy3-labeled DNA complexes. Bright-field, GFP and RFP images were recorded using the BD Pathway 435 System (BD Biosciences, Franklin Lakes, NJ, USA) which includes both the fluorescent and confocal microscopic sets as previously described (Liu et al., 2011). Excitation filters were set at 482/35 nm and 543/

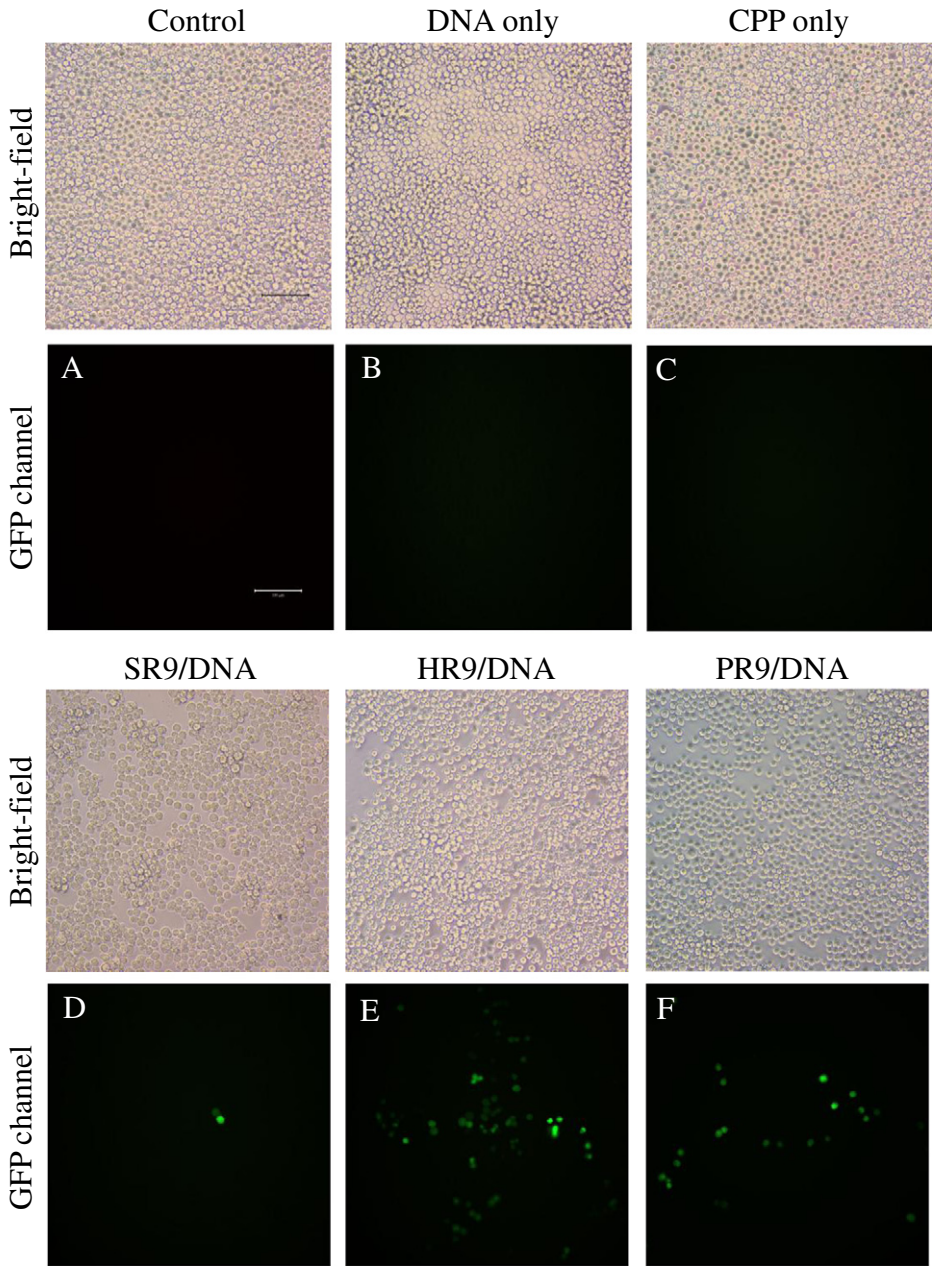
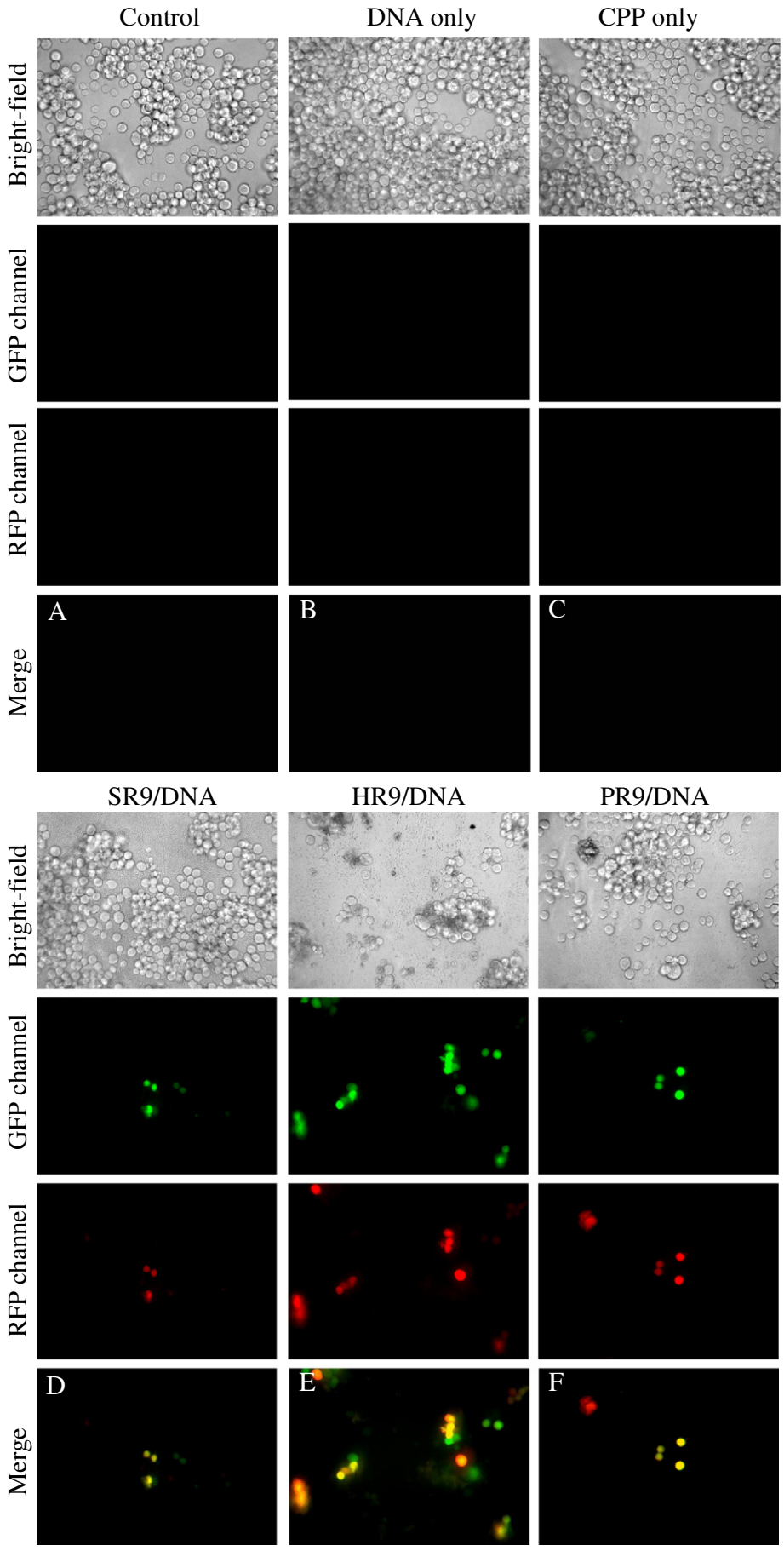


Fig. 3. Fluorescent microscopy of the CPP-mediated delivery of the pBacCecBEGFP plasmid DNA into Sf9 cells. Cells were treated with PBS as a control (A), the pBacCecBEGFP plasmid DNA only (B), HR9 only (C), SR9/DNA (D), HR9/DNA (E) or PR9/DNA (F) complexes. Images of bright-field and the RFP channel are shown from a fluorescent microscope at a magnification of 200×. The scale bar is 100 μm.

22 nm for green and red fluorescence, respectively. Emission filters were set at 536/40 and 593/40 nm for GFP and RFP channels, respectively.

For the functional gene assay, 3 μg of the reporter gene-containing plasmid DNA (pBacCecBEGFP or pBacCecBEGFP-3XP3DsRed2) were mixed with CPPs at the N/P ratio of 3 in PBS at a final volume of 60 μl and incubated for 2 h at room temperature. These CPP/DNA complexes were added to Sf9 cells and incubated for 30 min at 28 °C. After washing with PBS, cells were then supplemented with 1 ml of 10% serum-containing medium in each well and incubated at 28 °C for 72 h. Finally, medium was removed, and cells were washed twice with PBS gently. Fluorescent and bright-field images were observed using the BD Pathway 435 System (BD Biosciences) or the Olympus IX71 inverted fluorescent microscope (Olympus, Center Valley, PA, USA) as previously described (Lu et al., 2010). For the GFP channel, we set excitation at 460–490 nm and emission at 520 nm. For the RFP channel, we set excitation at 510–550 nm and emission at 590 nm. Images were captured using a RT3 2.0 Mp color CCD camera (Spot Imaging Solutions, Sterling Heights, MI, USA). The transfection efficiency of Sf9 cells was determined in two ways: 1) the

Fig. 4. Fluorescent microscopy of the CPP-mediated delivery of the pBacCecBEGFP-3XP3DsRed2 plasmid DNA into Sf9 cells. Cells were treated with PBS as a control (A), the pBacCecBEGFP-3XP3DsRed2 plasmid DNA only (B), SR9 only (C), SR9/DNA (D), HR9/DNA (E) or PR9/DNA (F) complexes. Images of bright-field, GFP and RFP channels are shown from a confocal microscope at a magnification of 200×. Overlap between EGFP and DsRed exhibits yellow color in merged GFP and RFP images.


Control
DNA only
CPP only
Bright-field
GFP channel
RFP channel
Merge
A
B
C
SR9/DNA
HR9/DNA
PR9/DNA
Bright-field
GFP channel
RFP channel
Merge
D
E
F

relative intensities of fluorescent images were converted and quantified using the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA) as previously described (Chang et al., 2005) and 2) digital image analyses were performed as previously described (Ogay et al., 2006).

2.7. Flow cytometric assay

To compare transfection efficiency, cells were treated according to procedures of the functional gene assay and analyzed using the Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) as previously described (Liu et al., 2008).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from Sf9 cells, and RT-PCR was conducted as previously described (Li et al., 2010) using the primers GFP5 (5′-AAGAATTCCATGGTGAGCAAGGGCGAGGAGCTGTT-3′) and GFP6 (5′-AAGAATTCTTAAGCTTGTACAGCTCGTCCATGCCGAGAGT-3′).

2.9. MTT assay

The MTT assay was conducted according to procedures of the functional gene assay to determine cell viability of Sf9 for 3 days as previously described (Chen et al., 2007).

2.10. Statistical analysis

Results were expressed as means ± standard deviations. Statistical comparisons between the control and treated groups were performed using the Student's *t*-test. Mean values and standard deviations were calculated for each sample examined in at least three independent experiments. The levels of statistical significance were set at $P<0.05$ (*) or 0.01 (**).

3. Results

3.1. Interaction of CPP and plasmid DNA

To reveal the interaction between arginine-rich CPP and DNA, the insect-specific pBacCecBEGFP plasmid was mixed with the increasing amounts of CPPs to form various N/P ratios (Fig. 1). We found that the relative mobility of DNA decreased when the ratio of CPP/DNA increased. The DNA-binding affinity differed among CPPs with the order of HR9 ($n=3$) > PR9 ($n=6$) = SR9 ($n=3$) (Fig. 1D). Plasmid DNA could be completely retarded with the increasing amounts of CPPs at ratios above 2.5 during electrophoresis. The N/P ratio of 3 was used as the combination ratio between CPP and plasmid DNA in subsequent experiments. These results demonstrated that CPP binds *in vitro* to plasmid DNA noncovalently.

3.2. CPP-mediated delivery of the Cy3-labeled DNA into Sf9 cells

To investigate the delivery of DNA by CPPs into insect cells, the pBlueScript-SK + plasmid DNA was first *in vitro* labeled with Cy3. The unlabeled and Cy3-labeled products were subjected to 1% agarose gel electrophoresis (Fig. S1). The successfully Cy3-labeled DNA appeared in red fluorescence in gel (Fig. S1B) and in yellow when the red color emitted by Cy3 dye is overlapping with the green color stained with SYBR Green I in gel (Fig. S1C).

In order to determine whether the Cy3-labeled plasmid DNA can be transported by three arginine-rich CPPs (SR9, HR9 and PR9) into insect cells or not, *S. frugiperda* Sf9 cells were treated with the Cy3-labeled DNA only, CPP only or CPP/Cy3-labeled DNA complexes (Fig. 2). No red fluorescence was detected in the cells treated with PBS (Fig. 2A), the Cy3-labeled DNA only (Fig. 2B) or CPP only (Fig. 2C) by a confocal microscope. In contrast, cells treated with SR9/Cy3-labeled DNA (Fig. 2D), HR9/Cy3-labeled DNA (Fig. 2E) or PR9/Cy3-labeled DNA (Fig. 2F) complexes showed red fluorescence at the RFP channel by a confocal microscope. These data indicated that arginine-rich CPPs (SR9, HR9 and PR9) could deliver the Cy3-labeled plasmid DNA into Sf9 cells.

3.3. CPP-mediated delivery of reporter gene-containing plasmid DNA into Sf9 cells

To explore the plasmid DNA can be delivered by CPPs and then the transferred DNA can be actively expressed in insect cells, an *EGFP* reporter gene-containing plasmid DNA was conducted for the functional assay at the protein level. Sf9 cells were treated with PBS, the pBacCecBEGFP plasmid DNA only, CPP only or CPP/pBacCecBEGFP complexes (Fig. 3). No green fluorescence was observed in the cells treated with PBS (Fig. 3A), the pBacCecBEGFP plasmid DNA only (Fig. 3B) or CPP only (Fig. 3C) by a fluorescent microscope. However, cells treated with SR9/DNA (Fig. 3D), HR9/DNA (Fig. 3E) or PR9/DNA (Fig. 3F) complexes exhibited green fluorescence at the GFP channel by a fluorescence microscope. These results demonstrated that arginine-rich CPPs (SR9, HR9 and PR9) are able to deliver the plasmid DNA into Sf9 cells, and the delivered DNA can be expressed subsequently.

To confirm the plasmid DNA can be transported and expressed in insect cells by CPPs, a dual reporter gene-containing plasmid DNA was performed for the functional assay. Sf9 cells were treated with PBS, the pBacCecBEGFP-3XP3DsRed2 plasmid DNA only, CPP only or CPP/pBacCecBEGFP-3XP3DsRed2 complexes (Fig. 4). In short, only the cells treated with SR9/DNA (Fig. 4D), HR9/DNA (Fig. 4E) or PR9/DNA (Fig. 4F) complexes possessed both green and red fluorescence at the GFP and RFP channels, respectively. These data demonstrated again that the plasmid DNA is able to be delivered by CPPs and be expressed in insect cells.

The transfection efficiency of Sf9 cells was determined from the digital image analyses at the GFP channel of Fig. 4 in two different ways (Chang et al., 2005; Ogay et al., 2006). We found that the efficiency of gene delivery differed among CPPs with the order of HR9 (6.67 ± 0.94%) > PR9 (4.00 ± 0.82%) > SR9 (3.33 ± 0.47%), when $n=3$ (Fig. 5). On the other hand, the order of transgenic efficiency of CPPs was calculated from the percentage of transfected cells over the total cell numbers as HR9 (6.16 ± 0.89%) > PR9 (3.90 ± 0.71%) > SR9 (0.19 ± 0.09%), when $n=5$.

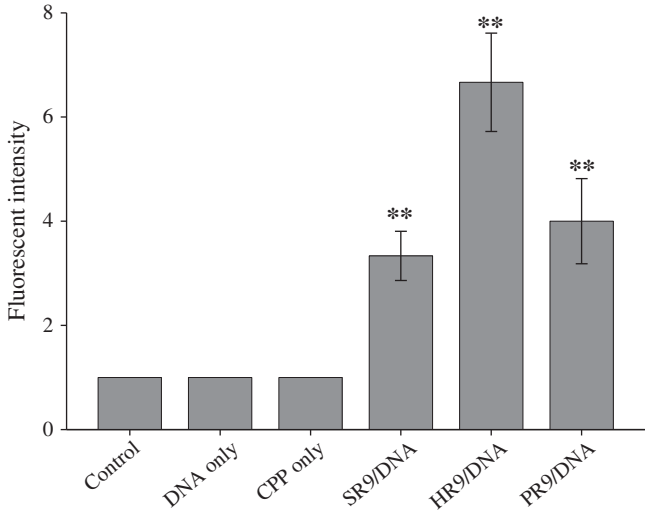


Fig. 5. DNA transfection efficiency based on the fluorescent intensity of the CPP-mediated delivery of the reporter gene DNA into Sf9 cells. Results were converted from fluorescent intensities at the GFP channel of Fig. 4 using the UN-SCAN-IT software. Significant differences were determined at $P<0.05$ (*) and $P<0.01$ (**). Data are presented as mean ± standard deviations from 3 independent experiments.

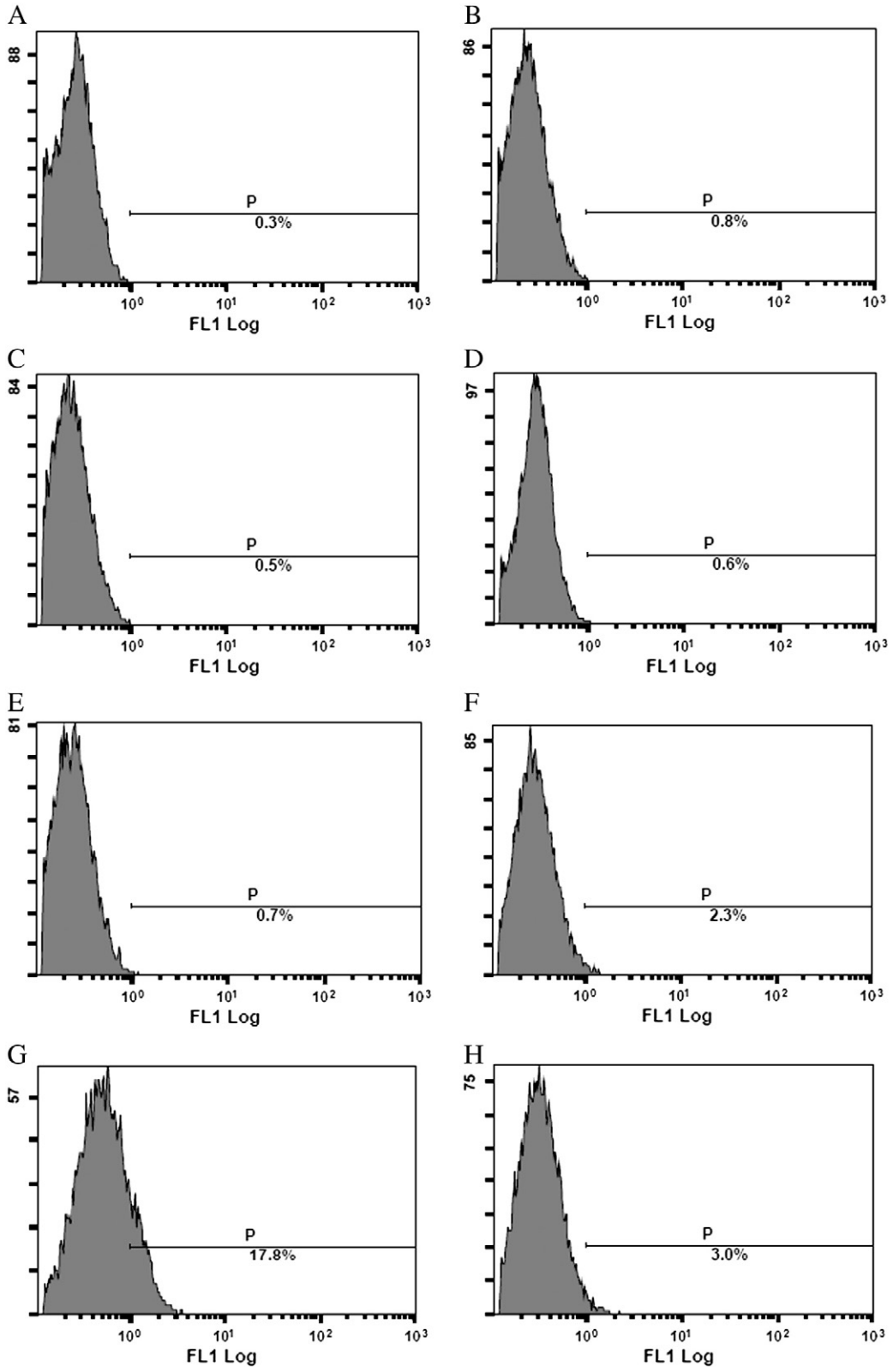


Fig. 6. Flow cytometric analysis of CPP-mediated DNA transfection in Sf9 cells. Profiles of insect cells treated with PBS (A), the pBacCecBEGFP plasmid DNA only (B), SR9 only (C), HR9 only (D), PR9 only (E), SR9/DNA (F), HR9/DNA (G) or PR9/DNA (H) are shown. Fluorescent images were analyzed using the Cytomics FC500 flow cytometer. Cell counts (*y* axis) at different fluorescent gate regions (*x* axis) were determined with the CXP software. Populations over the fluorescent gate region ($FL1 > 10^0$) are shown. (I) Comparative transfection efficiency derived from combined profiles (A–H). Significant differences were marked at $P<0.05$ (*) and $P<0.01$ (**). Data are presented as mean ± standard deviations from 3 independent experiments.

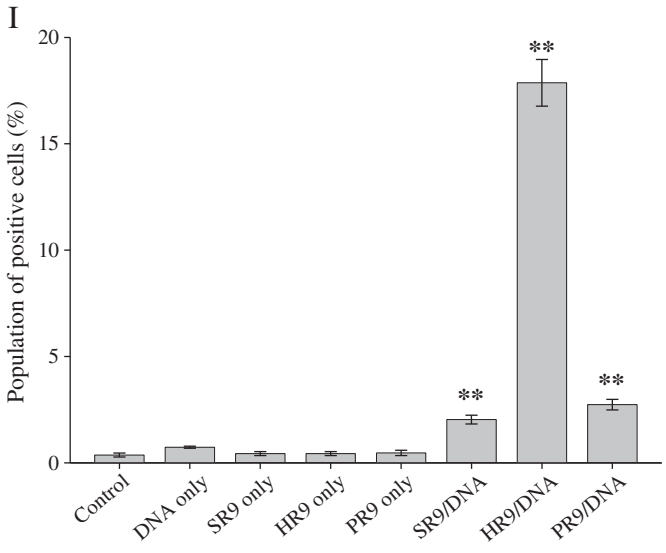


Fig.6 (*continued*).

3.4. Flow cytometric assay

To compare DNA transfection efficiency in the CPP-mediated gene delivery, Sf9 cells were treated with PBS (Fig. 6A), the pBacCecBEGFP plasmid DNA only (Fig. 6B), SR9 only (Fig. 6C), HR9 only (Fig. 6D), PR9 only (Fig. 6E), SR9/DNA (Fig. 6F), HR9/DNA (Fig. 6G) or PR9/DNA (Fig. 6H) complexes for the flow cytometric analysis. Cells treated with PBS, DNA, or CPP resulted in background fluorescence (Fig. 6A–E). However, the cells treated with SR9/DNA, HR9/DNA or PR9/DNA complexes exhibited green fluorescence (Fig. 6F–H). The efficiency of gene delivery varied among CPPs with the order of HR9 ($18.76 \pm 1.09\%$) > PR9 ($2.73 \pm 0.25\%$) > SR9 ($2.03 \pm 0.21\%$), when $n = 3$ (Fig. 6I). These results were consistent with those of fluorescent microscopy, indicating that CPPs are effective DNA carriers for intracellular delivery in insect cells.

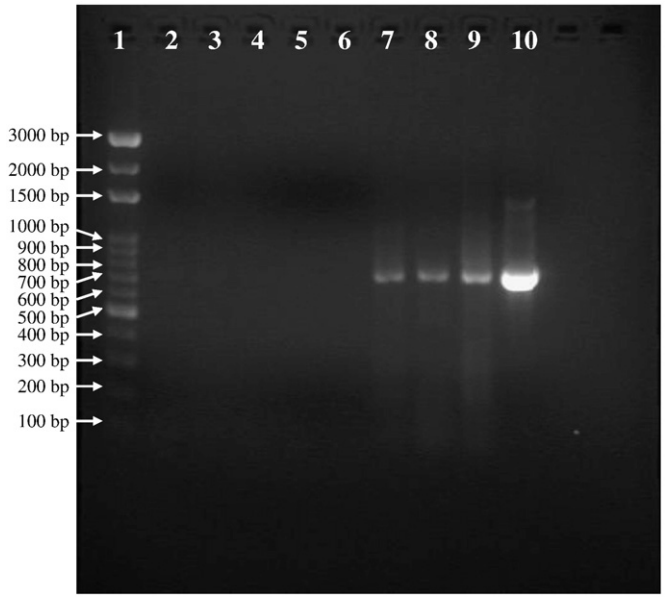


Fig. 7. RT-PCR analysis of *EGFP* transcripts in Sf9 cells treated with CPP/DNA complexes. Lane 1 is the 100 bp DNA ladder (Protech, Taipei, Taiwan). The RT-PCR products of cells treated with PBS (lane 2), the pBacCecBEGFP-3XP3DsRed2 plasmid DNA only (lane 3), SR9 (lane 4), HR9 (lane 5), PR9 (lane 6), SR9/DNA (lane 7), HR9/DNA (lane 8), PR9/DNA (lane 9) or a positive control (lane 10) were separated by DNA electrophoresis on a 2% agarose gel. The expected PCR product of the *EGFP* cDNA is about 743 bp amplified by both GFP5 and GFP6 primers.

3.5. RT-PCR

To confirm the plasmid DNA can be transported by CPPs and expressed in insect cells, the RT-PCR assay was conducted for the functional assay at the RNA level. Sf9 cells were treated with PBS, the pBacCecBEGFP-3XP3DsRed2 plasmid DNA only, CPPs only or CPP/pBacCecBEGFP-3XP3DsRed2 complexes (Fig. 7). A specific *EGFP* cDNA with 743 bp was amplified by both GFP5 and GFP6 primers from transcripts of cells treated with CPP/DNA complexes or the positive control (lanes 7–10). These data demonstrated the CPP-mediated intracellular gene delivery and expression at the RNA level.

3.6. Cytotoxicity assay

To evaluate any cytotoxicity caused by arginine-rich CPPs (SR9, HR9 and PR9), Sf9 cells were treated with PBS as a negative control, DNA only, CPPs only, CPP/DNA complexes or 70% alcohol as a positive control and subjected to the MTT assay (Fig. 8). Treatment of CPPs and CPP/DNA complexes showed cell viability of 84–93%. However, cells treated with 70% alcohol displayed significantly strong decrease in viability (9%). These results indicated that neither CPPs (SR9, HR9 and PR9) nor CPP/DNA complexes show cytotoxicity in Sf9 cells conspicuously.

4. Discussion

In recent years, CPPs were efficient and safe tools for gene transport. In this report, we demonstrated that arginine-rich CPPs can form stably noncovalent complexes with plasmid DNA *in vitro*. These CPP/DNA complexes formed at the optimized N/P ratio of 3 were able to enter into insect cells, and the delivered reporter gene-containing DNA was expressed at both the protein and RNA levels subsequently. Accordingly, high viability of Sf9 cells was shown in the treatment of CPPs or CPP/DNA complexes. Thus, arginine-rich CPPs possess the ability to deliver DNA intracellularly, and HR9 appears to be the most excellent carrier among arginine-rich CPPs tested here for gene delivery in insect cells.

In general, nonviral delivery systems including cationic liposomes and synthetic polymers have been developed as a means for gene delivery (Luo and Saltzman, 2000). In terms of biosafety, nonviral systems are relatively safer than viral delivery systems, but they often suffer from an unsatisfactory transfection efficiency (Nakase et al., 2010). CPPs possess the ability to not only efficiently internalize but also deliver cargos into various types of live cells (Dowdy and Snyder, 2005). The application of CPPs was recently introduced as an attempt to increase the gene delivery ability for nonviral systems. We found that arginine-rich CPPs were able to form stably noncovalent complexes with plasmid DNA (Fig. 1), and these CPP/DNA complexes could be transported and functionally expressed into cells (Figs. 2–6). These data are consistent with previous results obtained with the pentadeca-arginine peptide and DNA (Choi et al., 2006). They reported that the size of CPP/DNA complexes is about 400 nm initially. As the incubation time increased, CPP/DNA complexes grew larger, reaching 6 μm after one hour incubation. Their data further suggested that CPP/DNA complexes are present in the nuclei of human embryonic kidney 293T epithelial cells as associated complexes.

In addition to CPPs, transposon mutagenesis is another ideal chemical method of transfection techniques as it is based on a naturally occurring system in insect cells and has been widely used to transpose many insect species (Lynch et al., 2010). Especially, the *piggyBac* transposable element has been extensively studied as a useful tool in insect transgenesis recently due to its simplicity of movement and often high frequency of transformation (Ding et al., 2005; Lynch

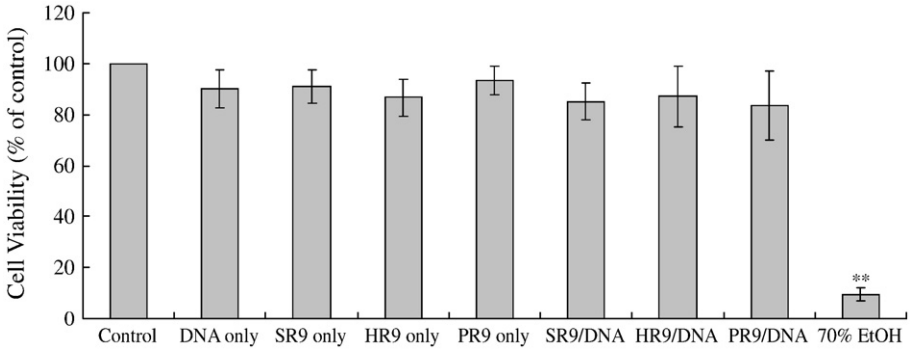


Fig. 8. Cell viability of the CPP/DNA treatment. Sf9 cells were treated with PBS as a negative control, 2.5 nM of the pBacCecBEGFP-3XP3DsRed2 plasmid DNA only, CPP only (15 μM of SR9, 7 μM of HR9 and 9.1 μM of PR9), CPP/DNA complexes or 70% alcohol (EtOH) as a positive control at a final volume of 200 μl for 10 min. The MTT assay was used to evaluate cytotoxicity 3 days after the treatment. Data are presented as mean ± standard deviations from 3 independent experiments. Significant differences were marked at $P<0.05$ (*) and $P<0.01$ (**).

et al., 2010). Drawbacks to use of the baculovirus expression system include the necessity for constant maintenance of baculovirus stocks, the need for fresh batch infections to be made each time and co-purification of recombinant baculoviruses or baculoviral proteins with HIV-1 Gag virus-like-particles. Therefore, *piggyBac* transposition was applied as a novel method to replace the baculovirus expression system and to create transgenic insects for continuous production of HIV-1 Gag virus-like-particles (Lynch et al., 2010). In addition, the *piggyBac* transposon was surprisingly discovered to be used for cancer gene discovery in mice (Rad et al., 2010). The genetically engineered transposon–transposase mice could be induced cancers whose type (hematopoietic *versus* solid) and latency were dependent on the regulatory elements introduced into *piggyBac* transposons. Analysis of 63 hematopoietic tumors revealed that *piggyBac* is capable of genome-wide mutagenesis in mice. We recently reported a special transposoduction strategy mediated by the nontoxic CPP-*piggyBac* transposase fusion protein to accomplish both protein transduction and mobile transposition in human cells (Lee et al., 2011).

Efficient DNA delivery plays a crucial role in gene therapy and transgenesis. A previous study claimed to obtain transfection efficiencies of at least 45% using polyethylenimine (PEI) form Fermentas Inc. in insect cell lines (Ogay et al., 2006). In contrast, we applied the jet-PEI reagent from Polyplus-transfection Co. (Wang et al., 2007) and found that the efficiency of gene delivery was only 0.20 ± 0.10% in Sf9 cells (unpublished data). To our knowledge, neither further application nor scientific article has appeared after the publication from Ogay et al. (2006). Additionally, PEI was considered as one of efficient currently-available transfection reagents, but this molecule is not biodegradable and relatively cytotoxic (Fischer et al., 1999; Forrest et al., 2003; Baoum and Berkland, 2011; our unpublished data). Transfection efficiencies of 6.67% (Fig. 5) and 18.76% (Fig. 6) may be criticized as unsatisfactory results in the present study. However, there are many parameters influencing the transfection efficiency of plasmid DNA in complex with CPPs, such as DNase degradation, ratio of CPP to DNA, conditions of CPP/DNA complex formation, mechanisms of CPP/DNA entry and transfection enhancers (Hellgren et al., 2004). In a previous study, we showed that SR9 could deliver DNA into plant cells (Chen et al., 2007). Both HR9 and PR9, two improved SR9 derivatives, provided the superior transfection efficiency over SR9 (Fig. 5). We reasoned that the differential CPP–DNA-binding affinities *in vitro* and the different mechanisms of cellular uptake of CPP/DNA are two limiting factors resulting in vary gene delivery efficiencies of these arginine-rich CPPs. Firstly, the differential CPP–DNA-binding affinities were well correlated with vary gene delivery efficiencies of CPPs (Figs. 1, 5 and 6). Secondly, our mechanistic studies indicated that the cellular entry for HR9/cargo complexes is mediated by the direct membrane translocation (Liu et al., 2011), while the cellular uptake for SR9/cargo is mediated by multiple internalization pathways (Hu et al., 2009). The mechanisms of CPP-mediated cellular uptake essentially influence the efficiencies and biological activities of delivered cargoes in cells (Nakase et al., 2010). Moreover, it was noted that the additional attachment of Mu DNA-binding domain (Xavier et al., 2009), LK15 peptide (Saleh et al., 2010) or stearylated nuclear localization signal peptide (Wang et al., 2011) to CPP, becoming Tat-NLS-Mu, Tat-LK15 or STR-NLS-R8 fusion peptides, significantly improved DNA transfer in cells or tumors. Interestingly, CPPs covalently combine with not only peptides but also noncovalently link with other devices can enhance DNA transfection efficiency. For instance, CPP-PEG-lipids could form self-assembled particles and could be applied in gene delivery (Maitani and Hattori, 2009). Recently, PEI-coated cationic magnetic iron nanoparticles mixed with plasmid DNA, followed by the addition of a CPP could increase gene transfer in cells or in the rat spinal cord (Song et al., 2010).

5. Conclusion

We demonstrated that arginine-rich CPPs (SR9, HR9 and PR9) are able to form stable complexes with plasmid DNA at the optimized N/P ratio of 3 and deliver DNA into insect cells in a noncovalent fashion. The delivered plasmids encoding the *EGFP* and *RFP* reporter genes could be expressed in cells. These CPPs and CPP/DNA complexes were proven to be not cytotoxic in insect cells. In conclusion, arginine-rich CPPs possess the ability of gene delivery in insect cells that may provide a useful tool for insect transgenesis.

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