Original Paper

Double-stranded DNA introduction into intact plants using peptide–DNA complexes

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Abstract Introducing exogenous genes into plant cells is an essential technique in many fields in plant science and biotechnology. Despite their huge advantages, disadvantages of current transfection methods include the requirement of expensive equipment, risk of gene damage, low transformation efficiency, transgene size limitations, and limitations of applicable plant types. Recently developed peptide-based gene carriers can deliver plasmid and double-stranded RNA. However, the delivery of double-stranded DNA (dsDNA), specifically PCR products, has not been studied. As dsDNA is handled in several plant science labs, peptide-based gene carriers are expected to be applicable to dsDNA in addition to plasmid DNA and double-stranded RNA. Here, we demonstrate dsDNA introduction into intact *Nicotiana benthamiana* leaves by using an ionic complex of a fusion peptide comprising (KH)$_9$ and Bp100 with dsDNA encoding *Renilla* luciferase as a reporter gene. The buffer condition for the complex preparation and infiltration significantly affected the transfection efficiency; this is because the structure of the complex in various protonated conditions contributed to the transfection efficiency. Structures of the complex and peptide are key factors for improving the peptide-based gene delivery system for plants.

Key words: Double-stranded DNA, gene carrier peptide, gene delivery.

Plant gene delivery systems, which are methods for introducing exogenous genes into plant cells, are essential in many areas of basic plant science and biotechnology (Chilton 2005). The *Agrobacterium*-mediated method (Broothaerts et al. 2005), particle bombardment (Boynton et al. 1988, Galbraith 2007), and polyethylene glycol method (Miranda et al. 1992) are well-established and practical plant transformation methods. Despite their advantages, their disadvantages include the requirement of expensive equipment, risk of gene damage, low transformation efficiency, transgene size limitations, and limitations of applicable plant species. Several studies demonstrate the use of cell-penetrating peptides to deliver plasmid DNA (pDNA) into permeabilized wheat embryos (Chugh and Eudes 2008), mung bean, and soy bean roots (Chen et al. 2007). Cell-penetrating peptides have also been used to deliver double-stranded RNA (dsRNA) to induce post-transcriptional gene silencing in tobacco suspension cells (Unnamalai et al. 2004). We recently developed pDNA delivery systems that utilize gene carrier peptides that contain a few functional domains (Numata et al. 2010, 2011, 2012, Numata and Kaplan 2010a, 2010b). We selected a fusion peptide that combines a copolymer of histidine and lysine (KH)$_9$ (18 amino acids) (Chen et al. 2000) with a cell-penetrating peptide termed Bp100 (KKLFKKILKYL) (Badosa et al. 2007) as an efficient gene carrier into intact plants (Figure 1). The polycation (KH)$_9$ can interact with the negatively charged nucleic acids via ionic interactions to form complexes (Osada et al. 2010). Meanwhile, Bp100 was originally designed and optimized to be an antimicrobial peptide against plant pathogens and destabilize cell walls and plasma membranes (Badosa et al. 2007). Thus, we developed a new system for introducing pDNA and dsRNA into intact leaves of *Arabidopsis thaliana*, *Nicotiana*

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dsDNA

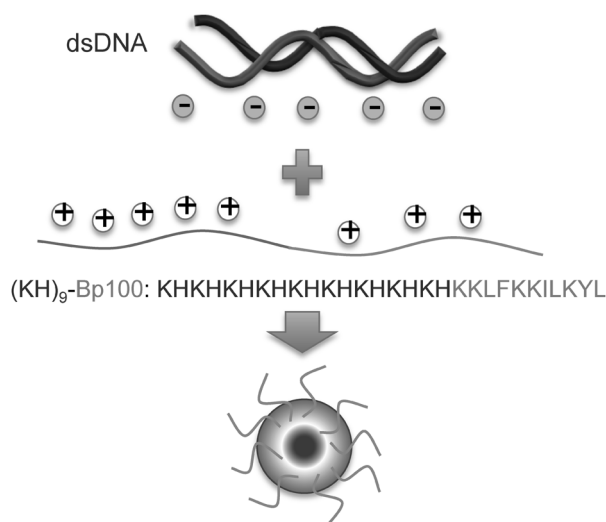$(KH)_9$-Bp100: KHKHKHKHKHKHKHKHKHKH KKLFKKILKYL

Figure 1.   dsDNA delivery system for plant cells using a gene carrier peptide. Negatively charged dsDNA is condensed by carrier peptide $(KH)_9$-Bp100 into dsDNA–peptide complexes. The carrier peptide comprises polycation, specifically lysine and histidine alternative 18mer $(KH)_9$, and cell-penetrating sequences (Bp100).

benthamiana, and *Populus* via infiltration (Lakshmanan et al. 2013, Numata et al. 2014). The peptide-based gene delivery system is not limited to the species of plants it can transform or transgene sizes. The peptide-based gene delivery system is not limited to the species of plants it can transform or transgene size, although the permeability and transfection efficiency needs to be improved. The limitation which the method actually faces is genetic materials to deliver.

The peptide-based gene carrier can deliver pDNA and dsRNA. However, the delivery of double-stranded DNA (dsDNA), specifically PCR products, has not been reported yet. As dsDNA is easy to be prepared by PCR and handled in many plant science labs, the peptide-based gene carrier is expected to be applicable to dsDNA in addition to pDNA and dsRNA. Here, we demonstrate dsDNA introduction into intact *N. benthamiana* leaves by using an ionic complex of the fusion peptide comprising $(KH)_9$ and Bp100 [$(KH)_9$-Bp100] with dsDNA encoding *Renilla* luciferase (RLuc) as a reporter gene. The transfection efficiency was almost the same as that using pDNA, indicating dsDNA can be used for peptide-based gene delivery into intact plants.

Materials and methods
Peptide synthesis
Fusion peptide $(KH)_9$-Bp100 (KHKHKHKHKHKHKHKHKHKH KKLFKKILKYL-NH$_2$, theoretical pI/Mw: 10.81/3810 Da) was custom synthesized at the Peptide Synthesis Facility, RIKEN Brain Science Institute, Wako, Japan by using standard 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis (Fields and Noble 1990). The peptides were purified using

high-performance liquid chromatography, and the molecular weights were determined by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

Plant preparation
N. benthamiana seeds were germinated in pots with planting medium containing a mixture of soil (Pro-Mix, Premier Tech Ltd., Canada) and vermiculite (2:1). Seedlings were grown in a plant incubator (Biotron NK System, Japan) with constant light and temperature (29°C) for 3 weeks.

Quantitative cytotoxicity studies of N. benthamiana *leaves*
To quantify leaf cytotoxicity towards buffers, *N. benthamiana* leaves were first infiltrated with buffers at various pH (MES buffer: pH 5.0, 6.0, and 7.4; PBS buffer: pH 7.4; HEPES buffer: pH 7.4 and 8.0) and concentrations (0.1, 1, 10, 30, 50, and 100 mM), followed by incubation for 12 h at 29°C with constant light. The light intensity was approximately 80 μmol photons $m^{-2} \cdot s^{-1}$. The leaves were sampled at 12 h by excising 1-cm-diameter discs around the infiltrated region by using a cork borer and stained with Evans blue solution as described previously (Guo and Crawford 2005). Briefly, the leaf discs were immersed in 0.1% w/v Evans blue solution and stained by three 5-min cycles of vacuum infiltration. The leaf discs were incubated in the Evans blue solution for another 30 min under vacuum and washed with PBS solution using three 15-min cycles of vacuum infiltration to remove excess and unbound stain. The leaf discs were subsequently immersed in 50% v/v methanol and 1% SDS solution, and heated at 60°C for 30 min to release the bound dye from the leaves. The supernatant was cooled at room temperature, and the absorbance was read at 600 nm using a multi-mode microplate reader (Spectra MAX M3, Molecular Devices Corporation, Sunnyvale, CA, USA). For the positive control (i.e., 100% cell death), leaf discs were heated at 100°C prior to staining. For the negative control (i.e., background absorbance readings), leaf discs infiltrated with deionized water were used for staining.

Preparation of dsDNA–peptide complexes
To prepare linear dsDNA encoding RLuc, the DNA fragment (2040 bp) containing the P35S promoter, RLuc gene, and nopaline synthase terminator (TNOS) sequences were amplified by PCR from the pBI221-P35S-RLuc-TNOS plasmid DNA template by using the following specific primers: M13F 5′-GTT TTC CCA GTC ACG ACG TTG T-3′ and M13R 5′-CAG GAA ACA GCT ATG ACC ATG ATT AC-3′ with KOD Plus Ver 2 DNA polymerase (Toyobo, Osaka, Japan). The PCR involved 30 cycles of pre-denaturation at 94°C for 2 min, denaturation at 98°C for 15 s, annealing at 64°C for 30 s, and extension at 68°C for 2 min. The amplification was confirmed by 1% agarose gel electrophoresis, and the target fragment was purified using the Qiagen Quick Gel Purification Kit (Qiagen, Hilden, Germany). To prepare dsDNA complexes, 10 μg ml^{-1} peptide was mixed with 0.1 μg dsDNA at an N/P ratio

of 0.1, 0.5, 1, 2, or 5 at 25°C; here, the N/P ratio refers to the ratio of the number of amines from peptide to the number of phosphates from DNA (Numata et al. 2012).

Chemical characterization of dsDNA–peptide complexes

The complexes were characterized by a zeta nanosizer (Zetasizer Nano-ZS, Malvern Instruments Ltd., Worcestershire, UK), which determined dimensions and zeta potentials. Solution containing dsDNA–peptide complexes was diluted to a final volume of 800 μl with deionized water (MilliQ) or buffers at various pH (MES buffer: pH 5.0, 6.0, and 7.4; PBS buffer: pH 7.4; HEPES buffer: pH 7.4 and 8.0). The buffers were all 30 mM. The zeta potential and zeta deviation of samples were measured 3 times by a zeta potentiometer, and averages were calculated by Zetasizer software v6.20 (Malvern Instruments Ltd.). Dynamic light scattering was performed with a zeta nanosizer using a 633-nm He–Ne laser at 25°C with a backscatter detection angle of 173° to obtain the hydrodynamic diameter (HD) and polydispersity index (PDI).

In order to observe the morphology of the dsDNA–peptide complexes by atomic force microscopy, the dsDNA–peptide complex solution was cast on freshly cleaved mica substrate and observed in air at 25°C. Atomic force microscopy (SPI3800/SPA 300HV, Seiko Instruments Inc., Chiba, Japan) observations were performed by using a silicon cantilever with a spring constant of 1.3 N m^{-1} in tapping mode as described previously (Numata et al. 2007). The cantilever tip-convolution effect was calibrated to obtain the true dimensions of the pDNA complexes as described previously (Numata et al. 2005, Numata et al. 2006).

Transfection efficiency of dsDNA–peptide complexes in N. benthamiana leaves

Approximately 100 μl dsDNA–peptide complex solution containing 0.1 μg dsDNA encoding RLuc prepared in deionized water or buffers (MES buffer: pH 5.0, 6.0, and 7.4; PBS buffer: pH 7.4; HEPES buffer: pH 7.4 and 8.0) was infiltrated directly into the abaxial section of fully expanded *N. benthamiana* leaves by using a 1-ml syringe without a needle. The treated plants were incubated at 29°C with constant light in a plant incubator for 12 h. The RLuc assay (Promega, Madison, WI, USA) was performed in quadruplicate to quantitatively evaluate RLuc gene expression according to manufacturer's protocol. Briefly, the leaves were sampled at 12 h by excising 10-mm-diameter discs around the infiltrated section by using a cork borer and lysed with RLuc assay lysis buffer (Promega). The lysate was briefly centrifuged, and the supernatant was mixed with RLuc assay substrate and RLuc assay buffer (Promega). Gene expression was evaluated on the basis of the photoluminescence intensity (in relative light units) by using a multi-mode microplate reader (Spectra MAX M3, Molecular Devices Corporation, Sunnyvale, CA, USA). The amount of protein in the supernatant was determined by BCA protein assay (Pierce Biotechnology, Rockford, IL, USA), and the

relative light units/protein weight (RLU mg^{-1}) was obtained.

Statistical analysis

The significance of differences in cytotoxicity studies using Evans blue staining and RLuc assay data were determined by unpaired *t*-tests with a two-tailed distribution. Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Evaluation of solvents for the peptide-based delivery system

Peptide–nucleic acid complexes were prepared in solution. The cytotoxicity of a reaction solution is an important factor for enhancing the transfection efficiency of the complexes into plants. First, we evaluated the cytotoxicity of the reaction solution, focusing on pH values. The cytotoxicity of 7 types of solvents (MES buffer: pH 5.0, 6.0, and 7.4; PBS buffer: pH 7.4; HEPES buffer: pH 7.4 and 8.0) and deionized water to *N. benthamiana* leaves were evaluated by using Evans blue staining (Figure 2). For the background absorbance readings, leaf discs infiltrated with deionized water were used, indicating that the cytotoxicity of the deionized water treatment was set to be 0% in Figure 2. The buffers at 5.0, 6.0 and 7.4 resulted in cell death ratios in the leaves <20% when the buffer concentration was <30 mM. Meanwhile, HEPES buffer at pH 8.0 resulted in the highest cell death ratio. As expected, cytotoxicity increased with increasing buffer pH and concentration. The infiltration of deionized water and the 30 mM
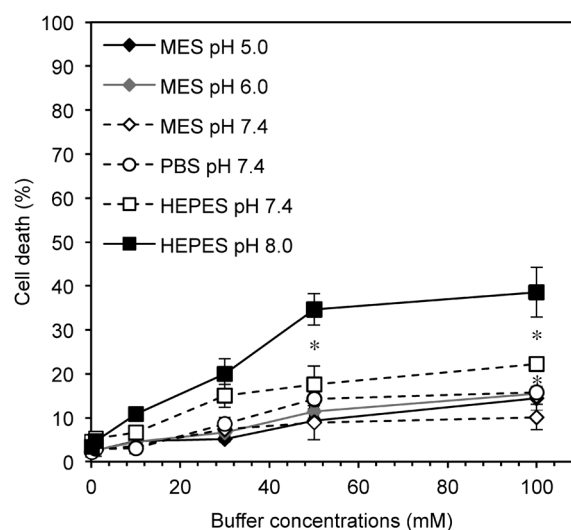

Figure 2. Quantitative cytotoxicity of buffers at various pH (MES buffer: pH 5.0, 6.0, and 7.4; PBS buffer: pH 7.4; and HEPES buffer: pH 7.4 and 8.0) and concentrations (0.1, 1, 10, 30, 50, and 100 mM) to *N. benthamiana* leaves assessed by Evans blue staining. Data are expressed as the percentage of cell death. Error bars indicate the standard deviation of samples (*n*=3). *Significant differences between groups at $p < 0.05$.

buffers at pH 5.0, 6.0 and 7.4 failed to cause significant cell death around the infiltrated area (Figure 3). Also, air infiltration without any solvents as a negative control to evaluate the effect of mechanical pressure by syringe was performed (Figure 3G). Based on the results, we decided deionized water and the 30 mM buffers at pH 5.0, 6.0, and 7.4 as appropriate solvents and used them for further experiments.

Physical properties of dsDNA–peptide complexes
To elucidate the function of the peptide-based dsDNA carrier, specifically the $(KH)_9$-Bp100 peptide (KHKHKHKHKHKHKHKHKHKKLFKKILKYL), dsDNA–peptide complexes prepared at different N/P ratios (0.1, 0.5, 1, 2, or 5) were analyzed with respect to size and surface charge. As mentioned above, the N/P ratio refers to the ratio of the number of amines from peptide to the number of phosphates from DNA.
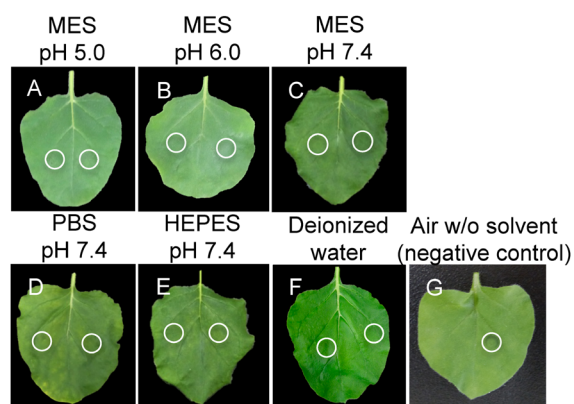

Figure 3. Cytotoxicity of solvents to *N. benthamiana* leaves. *N. benthamiana* leaves after infiltration of (A) 30 mM MES buffer (pH 5.0), (B) 30 mM MES buffer (pH 6.0), (C) 30 mM MES buffer (pH 7.4), (D) 30 mM PBS buffer (pH 7.4), (E) 30 mM HEPES buffer (pH 7.4), (F) deionized water, and (G) air infiltration (a negative control to see the effect of mechanical pressure by syringe). Each white circle denotes the infiltrated part.

The HD and the PDI of the complexes prepared at different N/P ratios in various solvents were measured by dynamic light scattering (Table 1). At lower N/P ratios (0.1 and 0.5), the complexes did not form homogeneously, resulting in a bimodal distribution of the diameters, i.e., relatively higher PDI. According to the hydrodynamic diameters, an N/P ratio exceeding 1 was required to prepare homogeneous complexes. The diameters of the complexes prepared at N/P of 0.1 and 0.5 in distilled water denotes that the peptide and DNA were aggregated, and then decreased with increasing N/P ratio, indicating more peptide molecules condense DNA more tightly. On the other hand, the diameters of the complexes prepared in the buffers were maintained independent of the N/P ratio. This indicates that the cationic peptide in the buffers did not condense DNA efficiently, even though the homogeneous complexes were formed.

To clarify morphology and sizes of the dsDNA–peptide complexes, complexes on mica substrate were observed by atomic force microscopy. Complexes prepared in deionized water at an N/P ratio of 1 exhibited a globular morphology with a diameter of approximately 100 nm (Figure 4). The diameter of the dsDNA–peptide complexes indicated that the dimensions determined by dynamic light scattering were larger than those determined by atomic force microscopy, because the complexes on mica substrate decreased approximately 80% in size due to drying in air (Numata et al. 2009).

Similar to the diameters, the zeta potentials, specifically the surface charges of the complexes were measured by a zeta nanosizer (Figure 5). The zeta potential of the dsDNA–peptide complex in deionized water was negative at N/P ratios of 0.1, 0.5, and 1, but became positive at an N/P ratio of 2 (Figure 5A). The complexes prepared in buffers also exhibited negative zeta potentials at low N/P ratios and positive zeta potentials at high N/P ratios such as 5, except PBS

Table 1. Hydrodynamic diameter (HD) and polydispersity index (PDI) of linear dsDNA complexes at various N/P ratios prepared in deionized water and various buffers.

Solvent	HD and PDI	N/P ratio				
		0.1	0.5	1	2	5
Deionized water	HD, nm	897±228	131±35, 1634±106	151±5	134±21	125±9.1
	PDI	0.82±0.21	—	0.37±0.02	0.35±0.04	0.45±0.02
MES pH 5.0	HD, nm	441±192, 3398±469	194±18, 4651±378	513±9	499±29	494±32
	PDI	—	—	0.28±0.03	0.29±0.06	0.27±0.06
MES pH 6.0	HD, nm	174±13, 1222±168	302±39, 3099±497	295±0.3	728±27	706±8.1
	PDI	—	—	0.39±0.05	0.25±0.04	0.29±0.01
MES pH 7.4	HD, nm	114±19, 1272±504	172±17, 1545±753	294±9.7	397±8.7	375±4.7
	PDI	—	—	0.31±0.02	0.28±0.03	0.26±0.05
PBS pH 7.4	HD, nm	507±106, 2656±210	98±9, 560±55	532±84	422±15	564±23
	PDI	—	—	0.55±0.08	0.28±0.03	0.31±0.07
HEPES pH 7.4	HD, nm	130±42, 1261±286	88±42, 924±313	229±4	386±13	371±13
	PDI	—	—	0.35±0.01	0.31±0.05	0.15±0.03

Dashes indicate no useful PDI values were obtained because of bimodal distribution.

buffer at pH 7.4 (Figure 5B). The complexes prepared under acidic conditions (pH 5.0) exhibited positive zeta potentials at a lower N/P ratio, specifically 2, which means that more protonated conditions provide more
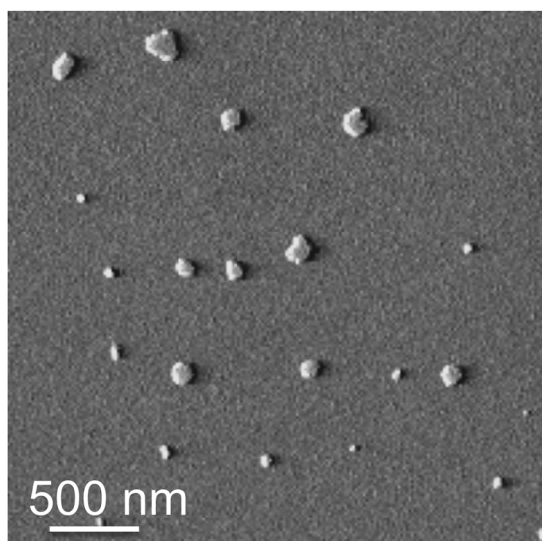

Figure 4.   Morphology of dsDNA–peptide complexes. Atomic force microscopic images of the dsDNA–peptide complexes prepared in deionized water at an N/P ratio of 1.

positive charges at the surface of the complexes.

Transfection via dsDNA–peptide complexes

To elucidate the transfection function of the dsDNA–peptide complexes, their transfection efficiency into intact *N. benthamiana* leaves was evaluated by RLuc assay; the dsDNA encoding RLuc was prepared and used for this assay. To determine the optimal N/P ratio of dsDNA–peptide complexes in deionized water, the delivery efficiency of dsDNA was quantitatively characterized by using dsDNA–peptide complexes prepared at different N/P ratios (Figure 6A). N/P ratios of 1 and 2 exhibited higher transfection efficiency than the others. Complexes prepared in deionized water at an N/P ratio of 1 showed a hydrodynamic diameter of 151 ± 5 nm and a zeta potential of approximately -30 mV. Complexes prepared in deionized water at an N/P ratio of 5 exhibited a positive zeta potential and slightly smaller sizes but exhibited significantly lower transfection efficiency. The transfection efficiencies of the complexes prepared in the buffers depended on not only the buffers, but pH as well, specifically protonated/deprotonated state (Figure 6B). Complexes prepared in PBS at pH 7.4 exhibited the highest transfection efficiency among all buffers, similar to that in deionized
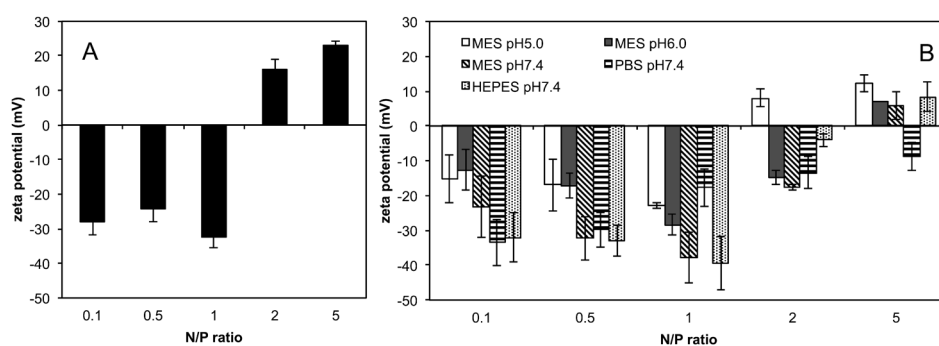

Figure 5.   Surface charges (i.e., zeta potentials) of dsDNA–peptide complexes prepared at different N/P ratios (0.1, 0.5, 1, 2, and 5) in (A) deionized water and (B) various buffers.
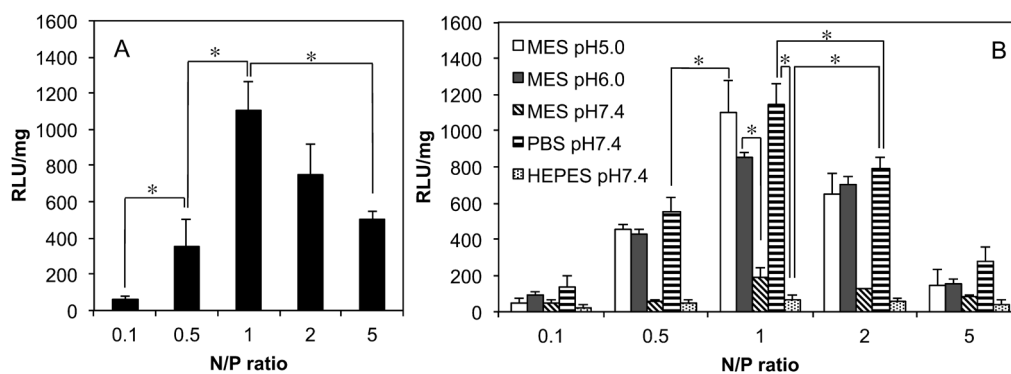

Figure 6.   Transfection efficiency of dsDNA–peptide complexes prepared at different N/P ratios (0.1, 0.5, 1, 2, and 5) 12 h after the injection of the complexes into *N. benthamiana* leaves according to RLuc assay results. (A) Transfection efficiency in deionized water. (B) Transfection efficiency in buffers (MES buffer: pH 5.0, 6.0, and 7.4; PBS buffer: pH 7.4; and HEPES buffer: pH 7.4 and 8.0). Error bars represent the standard deviations of samples ($n=4$). *Significant difference between 2 groups at $p<0.05$.

water (Figure 6A). The diameter and zeta potential of complexes prepared at an N/P of 1 in PBS at pH 7.4 were 532 ± 84 nm and -10 mV, respectively; these complexes were larger than those prepared in deionized water. However, their zeta potential was negative like the complexes prepared in deionized water at an N/P ratio of 1. These results indicate that the positive charge is not preferable for introducing dsDNA into intact leaves. Furthermore, the size of the complexes is not related to their transfection efficiency as long as they are in the sub-micrometer scale. The transfection efficiency of the dsDNA complex was similar to the transfection efficiency of the pDNA complex (approximately 1400 RLU/mg), according to our previous report (Lakshmanan, et al. 2013). This indicates that the linear dsDNA also formed ionic complexes with cationic peptides. However, we need to characterize the effect of molecular weight of DNA on transfection in the future work.

In summary, we propose the peptide-based dsDNA delivery system described herein as an easy and quick gene introduction technique. This method can theoretically be used without any special equipment or bacteria for any plant type when dsDNA–peptide complex solution can be infiltrated or introduced. Based on these advantages, we plan to expand our research target plants to practical plants rather than model plants such as *A. thaliana* and *N. benthamiana*. The transfection efficiency with dsDNA is almost same as that using pDNA (Lakshmanan et al. 2013), indicating dsDNA can be used for peptide-based gene delivery into intact plants. However, low transfection efficiency remains the primary drawback of the present method. Therefore, the transfection efficiency must be improved by using different peptides and plant tissues. The buffer condition for the complex preparation significantly affected the transfection efficiency in the present study. This is because the structure of the complex in various protonated conditions contributes to the transfection efficiency. Structural analysis of the complexes and homogeneous regulation of their structure are key factors for improving the present peptide-based gene delivery system for plants.

Acknowledgements

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