FEBS Letters 581 (2007) 1891–1897

Transfection and expression of plasmid DNA in plant cells by an arginine-rich intracellular delivery peptide without protoplast preparation

Chung-Pin Chen, Jyh-Ching Chou, Betty Revon Liu, Microsugar Chang, Han-Jung Lee*

Department of Life Science, National Dong Hwa University, Hualien 97401, Taiwan

Received 7 February 2007; revised 1 March 2007; accepted 21 March 2007

Available online 9 April 2007

Edited by Michael R. Sussman

Abstract **The delivery and expression of exogenous genes in plant cells have been of particular interest for plant research and biotechnology. Here, we present results demonstrating a simple DNA transfection system in plants. Short arginine-rich intracellular delivery peptide, a protein transduction domain, was capable of delivering plasmid DNA into living plant cells non-covalently. This peptide-mediated DNA delivery conferred several advantages, such as nuclear targeting, non-toxic effect, and ease of preparation without protoplast formulation. Thus, this novel technology shall provide a powerful tool to investigate gene function *in vivo*, and lay the foundation for the production of transgenic plants in future.**

Keywords: DNA transfection; Gene delivery; Green fluorescent protein; Intracellular trafficking; Protein transduction domain; Transgenic plants

1. Introduction

The first recombinant plant-derived pharmaceutical protein, human serum albumin, was initially produced in transgenic tobacco and potato plants [1]. The delivery and expression of recombinant DNA in living cells have proven invaluable in a wide variety of applications for basic plant research, plant biotechnology, and molecular farming. To achieve this goal, the first step requires an effective transport system to condense and deliver DNA into cells. Today, in general, plant biologists rely on two main processes for delivery and expression of exogenous genes in plants: stable genetic transformation and transient infection with viral vectors. With its extensive host range, *Agrobacterium*-mediated genetic transformation is the most widely used technology today for plant genetic engineering [2]. This soil bacterium possesses the natural ability to transform its host by delivering a tDNA (transferred DNA) of its Ti (tumor-inducing) plasmid into the host genome. Subsequently, replacing the bacterial genes with various genes of interest is the molecular basis for protocols of *Agrobacterium*-based plant gene transformation technology. More recently, several non-*Agrobacterium* species have been identified for plant gene transformation [3]. These bacteria are able to transiently and stably transform different plant tissues and species.

Virus-based vectors offer a reasonable alternative to *Agrobacterium* as a tool for transient gene expression in plant cells [4]. This may potentially result in the high yield of recombinant protein expression. However, viral vectors are rather limited by transgenic size and suffer in the low infection rate, for example, tobacco mosaic virus-based vectors [4]. In light of these concerns, many non-viral gene delivery systems have emerged as promising alternatives [5–7].

Protein transduction domains (PTDs) originally identified from the human immunodeficiency virus type 1 TAT protein are small (<20 residues) peptides containing many basic amino acids [8,9]. When PTD is synthesized within recombinant fusion protein or covalently cross-linked to a wide variety of cargoes, this cationic peptide can facilitate the uptake of biologically active macromolecules into mammalian cells [10–13]. PTDs can even facilitate systemic delivery of fused proteins to various tissues in a living mouse [14,15]. Protein transduction in plants was demonstrated by our group recently. We found that arginine-rich intracellular delivery (AID) peptide could effectively deliver not only covalently fused proteins into different types of plant cells [16], but also non-covalently mixed proteins into plant and animal cells [17,18], as well as animal tissues [17] in fully active forms. The observation of transducible PTD/DNA complexes was made by receptor-mediated delivery and persistent expression of a foreign gene to the liver of animals [19]. Subsequently, several reports have stated that PTDs afford the internalization of DNA/plasmids into mammalian cells [11,12,20,21]. We reported recently that AID peptide could non-covalently form stable complexes with short interfering RNA (siRNA) of enhanced green fluorescent protein (EGFP) and deliver these mixtures into cells [22]. After entry, siRNA appeared to stay in perinuclear regions within cell, and ultimately fulfilled its targeted *egfp* gene silencing.

The mechanism of cellular entry of PTDs in mammalian cells remains controversy. Several reports indicated that protein transduction delivering proteins into cells appears to be independent of classical endocytosis, energy, receptors, or active transporters [10,23,24]. Recent work has proposed that a specialized form of endocytosis, known as macropinocytosis, plays a major role in the uptake of PTD peptides and their cargoes [25–28].

*Corresponding author.
E-mail address: hjlee@mail.ndhu.edu.tw (H.-J. Lee).

Abbreviations: AID, arginine-rich intracellular delivery; Cy3, Cyanine 3; CytD, cytochalasin D; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide; NEM, *N*-ethylmaleimide; NLS, nuclear localization signal; PTD, protein transduction domain; R9, nona-arginine; SR9, synthetic nona-arginine

0014-5793/$32.00
doi:10.1016/j.febslet.2007.03.076

2. Materials and methods

2.1. Preparation of peptide/protein and plasmid DNA

Synthetic nona-arginine (SR9) peptide containing the nona-arginine (R9) sequence was *in vitro* synthesized and purified by HPLC described previously [18]. GFP and R9-GFP proteins were overexpressed and purified from *Escherichia coli* transformed with pQE8-GFP and pR9-GFP plasmids as previously described [16], respectively. pNLS-R9-GFP plasmid was generated by cutting pR9-GFP with *Nhe*I and *Bgl*II restriction enzymes, and followed by insertion of the DNA sequence encoding one copy of the nuclear localization signal (NLS) (sequence in single-letter amino acid code: PKKKRKV) from simian virus large T-antigen that were generated by annealing of primers NLS-1 (5′-CTAGCCCAAAGAAGAAGAGAAAGGTAA-3′) and NLS-2 (5′-GATCTTACCTTTCTCTTCTTCTTTGGG-3′). pHBT-sGFP(S65T)-NOS plasmid (GenBank Accession No. EF090408, kindly provided by Dr. Jen Sheen, Harvard University) contains a re-engineered *gfp* gene under the control of the 35S cauliflower mosaic virus (CaMV) enhancer fused to the basal promoter of the maize *C4PPDK* gene [29]. Plasmid DNA was purified with the Nucleobond AX100 Kit (Machery-Nagel, Duren, Germany).

2.2. Gel retardation assay

This experiment was carried out as described previously [30–32] with some modifications. Four micrograms of pEGFP-C1 plasmid (Clontech, Mountain View, CA, USA) was pre-mixed with various amounts of SR9 peptide (0, 0.4, 0.8, 1.2, 1.6, 2, and 4 μg) in phosphate buffered saline (PBS) to a final volume of 20 μl and incubated at 37 °C for 30 min in order to form peptide/DNA complexes with various molar nitrogen/phosphate (N/P) ratios (0, 0.2, 0.4, 0.6, 0.8, 1, and 2). Four micrograms of SR9 peptide was utilized without adding any DNA to represent maximal N/P ratio. Stoichiometric N/P ratio used in the DNA delivery mediated by cationic molecules was defined by the molar ratio between the cation group ($-NH_3^+$) and the anion group ($-PO_4^-$) [32]. These complexes were analyzed by electrophoresis on a 1% agarose gel in TBE (45 mM Tris–borate and 1 mM EDTA, pH 8.0) buffer at 100 V for 40 min, followed by staining with ethidium bromide for 30 min. Relative intensities of DNA bands were quantified by the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

2.3. AID peptide-mediated DNA delivery

Three different preparations [10 μg of SR9 peptide, 10 μg of pHBT-sGFP(S65T)-NOS plasmid, and 10 μg of SR9 peptide plus 10 μg of pHBT-sGFP(S65T)-NOS plasmid] were individually added in PBS to a final volume of 50 μl and incubated at 37 °C for 30 min. These preparations were transferred into the eppendorf tubes containing 350 μl of double deionized water, respectively. Plant material preparation was described previously [18]. Intact roots of mung bean (*Vigna radiata* L.) and soybean (*Glycine max* L.) were treated with solution consisting of either peptide alone or plasmid alone for 30 min at room temperature as controls. To test the plasmid DNA delivery into cells, plant roots were treated with peptide/plasmid mixtures for 30 min at room temperature. Roots were washed with water in order to remove free peptide/plasmid, and remained *in planta*. Roots were squashed onto slides before microscopy. The intensity of green fluorescence was measured at various time periods (5, 7, 15 min, 12, 24, 36, or 48 h) after treatment.

For experiments to determine optimal ratio between peptide and plasmid for DNA delivery, 10 μg of pHBT-sGFP(S65T)-NOS plasmid was pre-mixed with various concentrations of SR9 peptide in PBS to a final volume of 50 μl in order to form different N/P ratios (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5) and incubated at 37 °C for 30 min. Peptide/plasmid complexes were added to the eppendorf tube containing 350 μl of double deionized water. Plant cells were treated with various ratios of peptide/plasmid mixtures for 30 min at room temperature. The intensity of green fluorescence was measured 48 h after treatment. To investigate the potential mechanism, roots of mung bean were treated with SR9/pHBT-sGFP(S65T)-NOS mixtures in the presence or absence of 10 μM of cytochalasin D (CytD) (Sigma–Aldrich, St. Louis, MO, USA), 100 μM of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), 2 μM of valinomycin, 10 mM of sodium azide, 1 mM of *N*-ethylmaleimide (NEM), 10 μM of fusicoccin, or 2 μM of nigericin for 30 min at room temperature, respectively. For experiments at 4 °C, the protocol was conducted as described previously [16,18]. The relative intensities of fluorescence were quantified by the UN-SCAN-IT software.

2.4. In vitro labeling of plasmid DNA

Circular plasmid DNA was labeled with the *Label*IT Cyanine 3 (Cy3) Labeling Kit according to the manufacturer's instructions (Mirus Bio, Madison, WI, USA). Briefly, the *Label*IT Cy3 reagent and plasmid DNA [pHBT-sGFP(S65T)-NOS and pBluescript-SK+] were combined in Labeling Buffer A, followed by incubating at 37 °C for 1 h. The labeled plasmid DNA was separated from unattached label by ethanol precipitation using 1/10 volume of 5 M NaCl as the counter ion. For DNA localization assay, 10 μg of Cy3-labeled pHBT-sGFP(S65T)-NOS or pBluescript-SK+ plasmid DNA was pre-mixed with 10 μg of SR9 in PBS to a final volume of 50 μl and incubated at 37 °C for 30 min. SR9/Cy3-labeled plasmid DNA complexes were added to the eppendorf tube containing 350 μl of double deionized water. Plant roots were treated with either labeled plasmid DNA or SR9/labeled plasmid DNA complexes for 30 min at room temperature, and washed with water in order to remove free complexes. The intensity of red fluorescence was measured 48 h after treatment.

2.5. Confocal microscopy

Images were observed under an Eclipse E600 fluorescent microscope (Nikon, Melville, NY, USA) and recorded by a Penguin 150CL cooled CCD camera (Pixera, Los Gatos, CA, USA). Fluorescent images were observed by the TCS SL confocal microscope system (Leica, Wetzlar, Germany), and relative intensities of fluorescent images were quantified by the UN-SCAN-IT software as previously described [16,17].

2.6. Trypan blue and MTT assays

To examine the cytotoxicity of SR9 in plant cells, trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide (MTT) assays were performed in roots of mung bean as described previously [16,17] with some modifications. Fifty milligrams of root tissues of mung bean was treated with either water, 70% alcohol, or 20 μM of SR9 for 4 h at room temperature, followed by washing with water. Then 500 μl of MTT solution (0.5 mg/ml in water) was added. After 2 h of MTT treatment, MTT solution was removed. Then, 1 mL of dimethyl sulfoxide (DMSO) was added. After shaking for 1 h, DMSO solution was measured by a Multiskan EX microplate photometer (Thermo Electron, Waltham, MA, USA) at 600 nm wavelength [17].

2.7. Statistical analysis

Results were represented as means ± S.D. Statistical comparisons of the control with the treated group were performed by ANOVA. The level of significance was set at $P < 0.05$.

3. Results

3.1. Cellular entrance of AID peptide

To determine whether AID peptide itself enters into cells or stays outside during peptide-based cargo delivery, protein transduction assay was conducted [17,18]. Roots of mung bean (*Vigna radiata* L.) were treated with either GFP or R9-GFP fusion proteins. As shown in Fig. 1A, no or little green fluorescence was detected in roots incubated with GFP protein alone as a control. In contrast, root tissue incubated with R9-GFP protein (Fig. 1B and C) exhibited green fluorescence in a high efficiency. The signal was distributed evenly in almost all cells observed, and was predominantly located at higher levels in some nuclei (Fig. 1C). Roots incubated with NLS-R9-GFP protein showed the same nuclear targeting result with that of R9-GFP treatment (data not shown). Together, these results demonstrated that this AID peptide itself can enter into cells as a nuclear routing vehicle [33,34], and is the key component for peptide-mediated intracellular shuttle in plant cells.

3.2. AID peptide directly binds to plasmid DNA and delivers it into plant cells for gene expression

To test whether AID could bind to DNA *in vitro*, circular pEGFP-C1 plasmid was mixed with various concentrations