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Cellular internalization of arginine-rich peptides into tobacco suspension cells: a structure–activity relationship study

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Translocation of several fluorescently labeled arginine-rich peptides into intact plant cells was quantitatively examined in order to investigate the structural factors required for efficient cellular internalization, and thereby, to evaluate the potential of arginine-rich peptides as intracellular delivery vectors in plants. Cell-penetrating peptides (CPPs) such as arginine-rich peptides permit the direct introduction of biologically active macromolecules into plant cytoplasm to manipulate various intracellular processes. While a significant level of adsorption of applied arginine-rich peptides was observed in the cell walls rich in negative charges, removal of adsorbed peptides by trypsin treatment allowed determination of the amount of internalized peptides in a quantitative manner using spectrofluorometric analysis. The internalization of arginine-rich peptides depended on the number of arginine residues, and the peptide containing eight arginine residues showed most effective internalization. Besides, the position of small cargoes attached to the arginine-rich peptides markedly affected the internalization efficiency. The results obtained in this study provide useful information for the development of efficient intracellular delivery tools in plant science.

Keywords: cell-penetrating peptide; plant cell; arginine-rich peptide; fluorescence; cell wall

Introduction

Many intracellular biological processes are regulated by various proteinous macromolecules, and direct introduction of such regulatory-active molecules into cytoplasm is expected to provide a new strategy for elucidating the working mechanisms of the intracellular processes. However, these macromolecules are mostly hydrophilic and not capable of penetrating the plasma membrane, limiting the applicability of functional manipulation by exogenous administration. Recently, several peptides have been identified to have an ability to translocate across the plasma membrane even when macromolecules were attached to the peptides. These are referred to as cell-penetrating peptides (CPPs) and are used as intracellular delivery vectors for a large variety of cargoes such as peptides, proteins, nucleic acids, and even liposomes, which has received increasing attention for their applications to basic research as well as to therapeutics [1]. Of several classes of CPPs, those derived from proteins have been well characterized to date, including HIV-1 Tat (48–60) [2], penetratin from Antennapedia homeodomain protein [3], and *p*VEC from vascular endothelium cadherin [4]. Arginine-rich peptides, which are designed based on the fact that most of CPPs are composed of multiple arginine residues, have also been shown to be efficiently internalized into cytoplasm [5,6]. The internalization mechanisms of CPPs have been extensively studied, and both endocytic and direct translocation pathways have been proposed, which are expected to depend on the size of cargo molecules and the peptide concentration [7,8].

While the vast majority of studies concerning internalization of CPPs have been conducted using mammalian cells, several recent reports have demonstrated that CPPs can also be internalized into plant cells in a similar manner to mammalian cells [9–12]. However, these studies of plant cells use protoplasts, which lack cell walls, in most cases, and the effects of cell walls on internalization of CPPs into plant cells has not been examined. Cell walls, which represent fundamental differences between plant and mammalian cells, are composed of complicated network of various carbohydrates with occasional negative charges to form a characteristic milieu. Translocation of CPPs through plant cell walls depends on their physicochemical properties, such as molecular size and shape, and the distribution of positive charges in the molecule. Thus, such specific features inherent to plant cells may bring some structural requirements for internalization of CPPs, which are distinct from those against mammalian cells.

In this study, we investigated the structure–internalization activity relationship of arginine-rich peptides to explore structural factors important for internalization into plant cells. Several peptides having different number of arginine residues were synthesized, and their internalization efficiency was quantitatively measured using tobacco suspension-cultured cells. In addition to the effect of the number of arginine residues in a peptide, we also examined the effect of the position of cargo molecules in the arginine-rich peptide on internalization by comparison between peptides bearing a cargo molecule at the *N*- and *C*-terminal ends. A precise evaluation of internalization of arginine-rich peptides into intact plant cells provides important information about the structure of peptides required for effective internalization into plant cells, which opens up the development of new macromolecule delivery techniques to investigate the mechanisms of various intracellular events of plants.

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Materials and Methods

Plant Material

Tobacco suspension-cultured cells (BY-2) were used in this study. The cells were grown at 25 °C on a rotary shaker (100 rpm) in Linsmaier and Skoog (LS) medium (pH 5.8) supplemented with 10 μM 1-naphthalenacetic acid, 1 μM 5-benzyladenine, and 3% sucrose. They were subcultured every week and used for 3–4 days after subculture.

Peptide Synthesis and Fluorophore Labeling

Arginine-rich peptides were synthesized and labeled with fluorescein diacetate (FDA) according to Ref. 6 (Figure 1). Briefly, all peptides (GC-amide, RRRRGC-amide, RRRRRRRRGC-amide, RRRRRRRRRRRRGC-amide, SASAGLQIAC-amide, and CGRRRRRRRR-amide) were synthesized by Fmoc solid-phase peptide synthesis on a Rink amide resin (Advanced ChemTech, Louisville, KY). After purification by RP-HPLC, each peptide was labeled by treatment with 5-maleimidofluorescein diacetate (3 eq) in 30% methanol/DMF for 3 h. The labeled peptides were purified by RP-HPLC. Electrospray ionization quadrupole mass spectrometry (ESI-MS, LCMS-2010, Shimadzu, Kyoto, Japan) was employed to confirm the molecular mass of the desired products (Table 1).

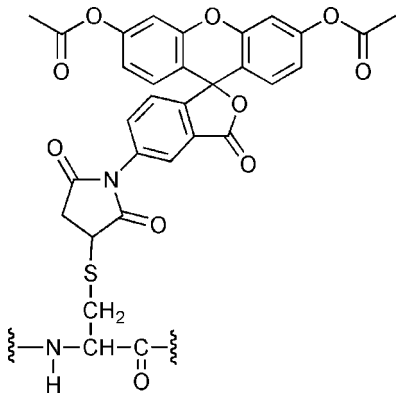

Figure 1. Structure of FDA-labeled cysteine residue.

Table 1. Structure of peptides used in this study

Peptide	Structure	Molecular mass (Da) Calcd.	Obsvd.
R4-FDA	FDA | RRRRGC-amide	1313.4	1312.9
R8-FDA	FDA | RRRRRRRRGC-amide	1938.2	1938.1
R12-FDA	FDA | RRRRRRRRRRRRGC-amide	2562.9	2562.3
Cont-FDA	FDA | SASAGLQIAC-amide	1430.5	1430.0
FDA-R8	FDA | CGRRRRRRRR-amide	1938.2	1938.3
GC-amide	FDA | GC-amide	688.7	688.1

Microscopic Analysis of Cellular Internalization

Tobacco suspension-cultured cells were collected by filtration through Miracloth (Calbiochem, San Diego, CA). These cells were resuspended in the buffer (2.0 mM MES, pH 5.75) in a microfuge tube (20 mg/ml) and incubated for 30 min at 25 °C using a tube rotator (Biospin MBS-1, Tokyo Rikakikai, Tokyo, Japan). Aliquots of cell suspensions (90 μl) were mixed with 10 μl of sample solutions dissolved in distilled water and incubated for 30 min at 25 °C using the tube rotator. The cell suspensions were then centrifuged for 4 min at 180 × *g* and the supernatant was removed. The cells were washed twice with the buffer and subjected to fluorescence microscopic analysis (BX51N-33-FLD-2, Olympus, Tokyo, Japan) at excitation wavelength of 486 nm and emission wavelength of 520 nm. For measurement of the internalization into plasmolyzed cells, cells were treated with 10% sorbitol for 10 min prior to sample addition.

Quantitative Measurement of Cellular Internalization by Spectrofluorometric Analysis

Tobacco cells collected by filtration through Miracloth were resuspended in the buffer (2.0 mM MES, pH 5.75) in a microfuge tube (40 mg/ml) and incubated for 30 min at 25 °C using the tube rotator. Aliquots of cell suspensions (180 μl) were mixed with 20 μl of sample solutions dissolved in distilled water and incubated for 30 min at 25 °C using the tube rotator. The cell suspensions were then filtered through Cosmospin filter H (Nacalai Tesque, Kyoto, Japan) by centrifugation at 100 × *g*, and the cells were washed twice with the buffer. The cells were treated with trypsin (0.6 mg/ml) for 30 min at 37 °C and washed twice with the buffer. After the cells were incubated with CelLytic P (200 μl, Sigma, St. Louis, MO) for 30 min, the solution was filtered through Cosmospin filter H by centrifugation at 400 × *g*. The filtrate was subjected to spectrofluorometric analysis (RF-1500, Shimadzu) at an excitation wavelength of 486 nm and an emission wavelength of 520 nm.

Results and Discussion

Microscopic Analysis of Internalization of Arginine-rich Peptides into Tobacco Cells

In this study, we used tobacco suspension cells for evaluation of cellular internalization of arginine-rich peptides. Tobacco is an important model plant used to study plant–pathogen interactions, and thus, direct introduction of macromolecules into cytoplasm by means of CPPs can increase our understanding of plant defense system.

We first analyzed the internalization of R8-FDA peptide, in which the *C*-terminal cysteine residue was fluorescently labeled with FDA (Figure 1, Table 1), into tobacco suspension cells using fluorescence microscopy. As shown in Figure 2, significant fluorescence was observed in cells treated with 10 μM R8-FDA. The cells treated with Cont-FDA that lacked arginine residues showed no fluorescence. Accumulation of R8-FDA in nuclei was also observed as shown in previous reports [9,12]. In addition, an intense fluorescence was observed in the cell walls when treated with R8-FDA, suggesting that a large amount of R8-FDA was adsorbed in the cell wall. When the cells were treated with lower concentration of R8-FDA (1 μM), adsorption was still observed (Figure 2). The adsorption of arginine-rich peptides to the cell walls was also observed in previous studies using plant tissues [9], although no particular attention has been paid for this

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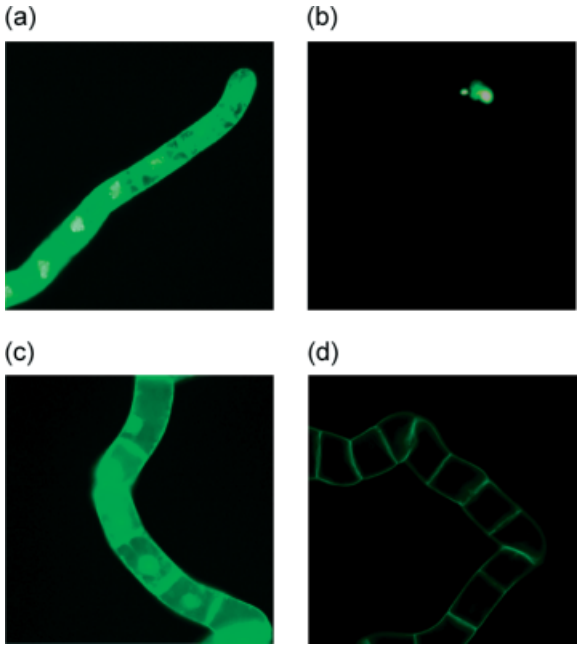

Figure 2. Fluorescence microscopic analysis of tobacco cells treated with 4.8 μM FDA (a), 10 μM Cont-FDA (b), 10 μM R8-FDA (c), and 1 μM R8-FDA (d).

phenomenon. The cell walls contain free carboxylate groups of galacturonic acid moieties in the pectin layer, and therefore, it is very likely that the positive charges of arginine side chains strongly interact with the negative charges in the cell walls via electrostatic interactions. Similar inhibitory effects of acidic materials on the internalization of arginine-rich peptides have also been observed in the experiments using mammalian cells [13]. When arginine-rich peptides were incubated with mammalian cells in the presence of serum, the internalization efficiency was significantly decreased, because of the reduction of their effective extracellular concentrations by the tight binding with albumin, a major acidic protein in serum.

To confirm that the adsorption of the arginine-rich peptides to the cell walls actually occurs, the cells were plasmolyzed before treatment with the peptides and observed by fluorescence microscopy (Figure 3). The cells treated with R8-FDA showed a significant fluorescence in the cell walls as well as in the cytoplasm. In contrast, no fluorescence was observed in the cell walls by the treatment with FDA alone, while the cytoplasm showed a definite level of fluorescence as a consequence of internalization by simple diffusion. These results clearly indicate that the internalization

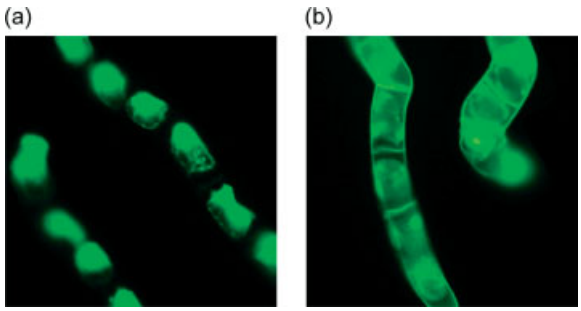

Figure 3. Fluorescence microscopic analysis of plasmolyzed tobacco cells treated with 4.8 μM FDA (a) and 10 μM R8-FDA (b).

efficiency of arginine-rich peptides is affected by the negative charges in the cell walls.

Thus, to accurately quantitate the internalization of FDA-bound arginine-rich peptides, the peptides adsorbed in the cell walls had to be removed before spectrofluorometric analysis. Considering the successful removal of arginine-rich peptides adsorbed on the plasma membrane by trypsin treatment for both mammalian [14] and plant cells [15], the tobacco cells were treated with trypsin after incubation with the testing peptides in the present study. As shown in Figure 4, trypsin treatment was quite effective in removing the peptides adsorbed on the cell walls as well as on the plasma membranes, which allowed the selective quantitation of the fluorescently labeled peptides present in the cytoplasm.

Quantitative Evaluation of Internalization of Arginine-rich Peptides into Tobacco Cells

We then quantitatively evaluated the amount of arginine-rich peptides internalized into tobacco cells. The cells were incubated with the peptides and treated with trypsin. To release the peptides present in the cytoplasm, the cells were treated with a non-ionic detergent-based lysis reagent, and the fluorescence intensity in the filtrates was measured by a spectrofluorometer.

As shown in Figure 5, the amount of internalized R8-FDA increased in proportion to its concentration, and was not saturated even at 20 μM. Similar concentration dependence has

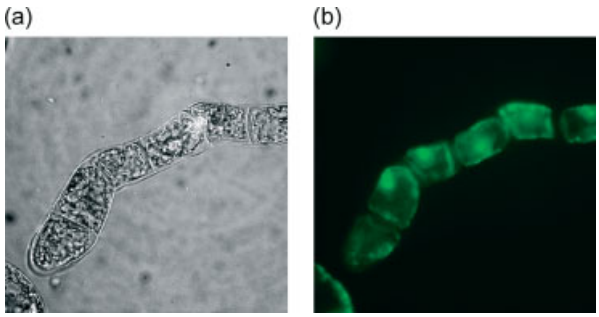

Figure 4. Fluorescence microscopic analysis of tobacco cells treated with trypsin after incubation with 10 μM R8-FDA. (a) Optical image and (b) fluorescence image.

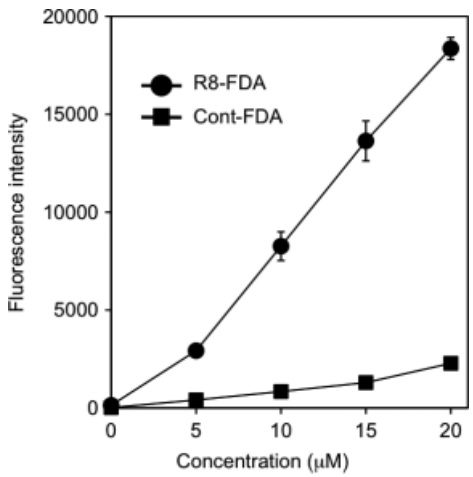

Figure 5. Effect of concentrations on cellular internalization of R8-FDA (circle) and Cont-FDA (square). Data points are the mean ± SD of triplicate measurements. For some data points, error bars are too small to be visible.

been observed for the internalization of Tat, another cationic CPP, into triticale mesophyll protoplasts [10]. Almost no internalization was observed for Cont-FDA even at 20 μM. Coefficients of variation for the detection at each concentration were less than 10%, indicating that the method developed in this study allows highly accurate quantitation.

Effect of Length of Arginine-rich Sequence on Internalization

Next we examined the effect of the number of arginine residues in a peptide on internalization into tobacco cells. Among four peptides that contain 0, 4, 8 or 12 arginine residues, R8-FDA showed the highest internalization efficiency (Figure 6). No internalization was observed for GC-FDA that lacked arginine residues. This result also confirmed that the peptide fragments produced by trypsin digestion did not penetrate into cells by simple diffusion. Internalization of R12-FDA was not as efficient as expected from the studies using mammalian cells, in which peptides composed of 12 arginine residues showed higher internalization efficiency than those of 8 arginine residues [13]. This is probably due to the difference in the extent of adsorption into the cell walls between R8-FDA and R12-FDA. It is likely that the latter binds to the cell walls more tightly and is not easily released into the apoplastic space, resulting in a decrease in its effective concentration for internalization. As mentioned above, a similar interference by adsorption to the additional components in the medium has also been observed in the study using mammalian cells [13], in which a significant decrease in internalization was observed particularly for peptides containing 12 and 16 arginine residues as a consequence of strong adsorption to a serum protein.

On the other hand, R4-FDA showed considerable internalization into tobacco cells in the present study when compared with that into mammalian cells [6,13]. R4-FDA is also attracted by the negative charges of the cell walls, but its interaction could be weaker than that of R8 and R12. Thus, for the case of R4-FDA, the cell walls might have an enrichment effect that increases the peptide concentration in the apoplastic space to facilitate the internalization. It is also possible that differences in the composition of the plasma membrane between plant and mammalian cells affect the internalization efficiency of R4-FDA.

Thus, the plant cell walls have appreciable effects on the internalization of arginine-rich peptides into plant cells. When choosing an optimum number of arginine residues for efficient internalization of arginine-rich peptides into plant cells, we must consider two conflicting requirements, namely efficient penetration across the plasma membrane and avoidance of adsorption to the cell walls. As a compromise, an optimum of around eight seems to occur as to the number of arginine residues for effective internalization into plant cells.

Effect of the Cargo Position in Arginine-rich Peptides on Internalization

For delivery of various cargo molecules into cells using arginine-rich peptides, it is possible to attach the cargo to arginine-rich peptides either at the *N*- or *C*-terminal end. However, the effect of cargo position on cellular internalization of arginine-rich peptides has not been examined in detail. In this study, we individually prepared the arginine-rich peptides containing a cysteine residue labeled with FDA at the *N*- or *C*-terminus, and compared their internalization efficiency to investigate the influence of the cargo position on internalization. As shown in Figure 7, the peptide bearing the cargo at the *C*-terminus showed 4 times higher internalization efficiency than that at the *N*-terminus. This result indicates that the position of cargo molecules can affect the internalization efficiency of arginine-rich peptides. Similar differences with respect to the cargo position were observed for the internalization of transportan 10 (TP10), an amphipathic CPP that contains no arginine residues, into mammalian cells [16]. Although the reason why the position of cargoes in the peptide affects the internalization has not yet been elucidated, TP10 bearing a cargo molecule at the *N*-terminal end exhibited the lower internalization efficiency than that attached to the central region of the peptide. It has been reported that cationic CPPs internalize into cells by endocytic uptake including macropinocytosis or by direct membrane translocation [8]. For the case of cationic CPPs with relatively small cargoes, however, it is suggested that their internalization into cells mainly occur only via direct membrane translocation, as is proposed for that of TP10 [17,18]. Considering the fact that the effect of cargo positions on internalization efficiency is similar between arginine-rich peptides

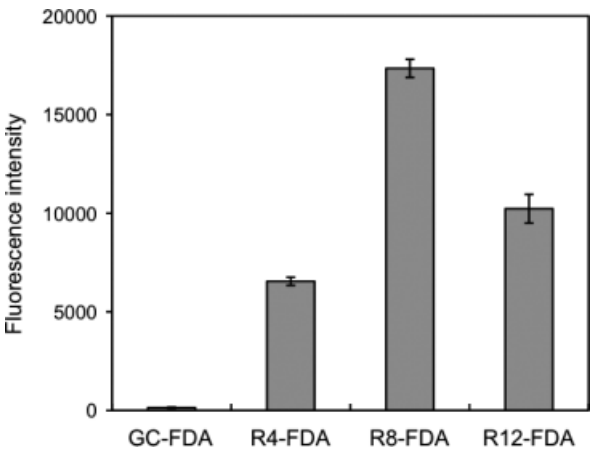

Figure 6. Effect of the number of arginine residues on cellular internalization. Cells were treated with each peptide at 10 μM. Data points are the mean ± SD of triplicate measurements.

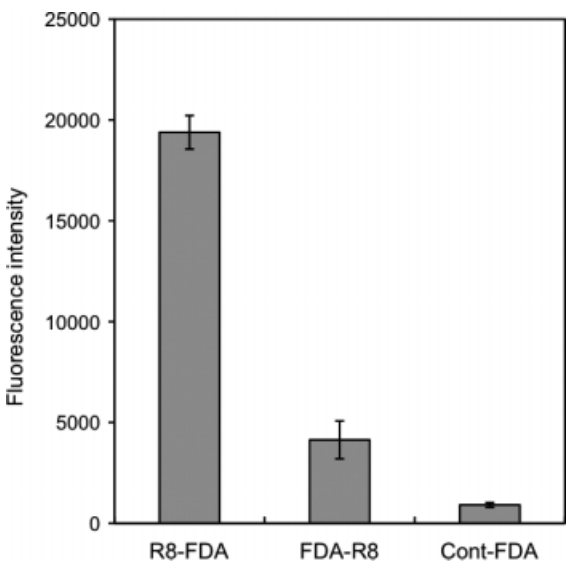

Figure 7. Effect of cargo positions in arginine-rich peptides on cellular internalization. Data points are the mean ± SD of triplicate measurements.

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and TP10, it is possible that the arginine-rich peptides used in this study also directly translocate into cells in a manner similar to that of TP10. Since no difference in cargo position-dependent efficiency of internalization by CPPs has been reported for larger cargo molecules such as proteins, the effect of cargo positions might be limited to work on small cargo molecules.

This study has demonstrated that small cargo molecules should be attached to the *C*-terminal end of arginine-rich peptides for their efficient internalization. Further investigations to clarify the molecular mechanisms underlying this phenomenon are required for establishing basic principles for controlling the cargo delivery into plant cells as well as mammalian cells.

Conclusion

In this study, we have succeeded in quantitative evaluation of internalization of arginine-rich peptides into plant cells. Since plant cells are surrounded by the cell walls rich in negative charges, significant adsorption of the peptides therein was observed. After removal of the adsorbed peptides by trypsin treatment, the accurate amount of internalized peptides was determined by spectrofluorometric analysis. The established method allowed understanding of the effect of the number of arginine residue on internalization into intact plant cells, and revealed that the peptide containing eight arginine residues showed the most effective internalization. We also found that the position of cargoes markedly affected the internalization efficiency. The information obtained in this study will be useful for future construction of efficient intracellular delivery tools in plant sciences.

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