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Cellular uptake of cell-penetrating peptides *p*VEC and transportan in plants‡

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Abstract: Internalization of fluorescently labeled CPPs, *p*VEC, transportan and scrambled *p*VEC, in a range of plant cells was investigated. Cellular uptake of the peptides was found to be tissue dependent. *p*VEC and transportan were distinctly internalized in triticale mesophyll protoplasts, onion epidermal cells, leaf bases and root tips of seven-day old triticale seedlings but showed negligible florescence in coleoptile and leaf tips as observed under a fluorescence microscope. Further, *p*VEC and transportan uptake studies were focused on mesophyll protoplasts as a system of investigation. In fluorimetric studies transportan showed 2.3 times higher cellular internalization than *p*VEC in protoplasts, whereas scrambled *p*VEC failed to show any significant fluorescence. Effect of various factors on cellular internalization of *p*VEC and transportan in protoplasts was also investigated. The cellular uptake of both the peptides was concentration dependent and nonsaturable. The cellular uptake of *p*VEC and transportan was enhanced at low temperature (4 °C). The presence of endocytic/macropinocytosis inhibitors did not reduce the cellular uptake of the peptides, suggesting direct cell penetration, receptor-independent internalization of *p*VEC and transportan into the plant cells. Copyright © 2007 Crown in the right of Canada. Published by John Wiley & Sons, Ltd.

Keywords: cellular internalization; cell-penetrating peptide; plant cell/tissue

INTRODUCTION

Cell-penetrating peptides have emerged as a class of short peptides with a property to translocate across cell membranes. The distinct ability of CPPs to deliver macromolecules that are otherwise rendered impermeant by the cell membranes has led to the development of novel peptide-mediated gene and protein delivery methods in human diagnostics and therapeutics [1,2]. The process of cellular internalization of CPPs still remains a controversial subject, as some reports suggest that the cellular uptake of CPPs is receptor-independent and nonendocytic [3–7], whereas others indicate that these peptides gain entry into the cell via endocytosis/micropinocytosis-mediated pathways [8–10].

The majority of the structural and functional studies on CPPs are based on mammalian cell lines. Plant systems largely remain unexplored in this context except some recent reports focusing on arginine-rich CPPs in plants. Polyarginine (R12) has been shown to deliver dsRNA to tobacco suspension cells to induce post-transcriptional silencing of *gus* and *npt*II genes [11]. Fluorescent proteins have been delivered in plant cells either by fusion or noncovalent interaction with arginine-rich intracellular delivery (AID) peptides [12,13]. We have also shown that monomer and dimer of HIV-1 Tat basic domain translocate across plasma membrane and accumulate in the nuclei of triticale mesophyll protoplasts [14]. Only one report for the cellular internalization of non-arginine-rich CPPs in tobacco protoplasts has come to light [15], although carrier functions of these peptides have been well demonstrated in animal system [16–18]. Therefore, keeping in view the potential importance of non-arginine-rich CPPs in cellular studies as well as in cargo delivery, we investigated the uptake of *p*VEC and transportan in plant cells. *p*VEC, a 18-amino-acid peptide is derived from the murine vascular endothelial-cadherin protein [16]. Transportan is a 27-amino-acid long synthetic CPP with *N*-terminal sequence built from the fragment of neuropeptide galanin linked through a lysine residue to mastoparan [17,19].

In the present study, we used visual fluorescence microscopy to investigate the uptake of fluorescently labeled *p*VEC and transportan in plant cells ranging from single, wall-less triticale mesophyll protoplast to actively dividing leaf bases and root tips. Scrambled *p*VEC served as negative control in all the experiments. Further, fluorimetric analysis was carried out to study the effect of different factors (concentration, temperature and endocytic/macropinocytic inhibitors) in mesophyll protoplasts.

Abbreviations: CPP, cell-penetrating peptide; CPW, cell protoplast wash solution; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; FDA, fluorescein diaccetate; *p*VEC, peptide vascular endothelial-cadherin.

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MATERIALS AND METHODS

Preparation of Plant Material

Mesophyll protoplast isolation and purification was carried out under aseptic conditions from six-day-old Triticale cv AC Alta seedlings as described by Chugh and Eudes (2007) [15]. The final protoplast density was adjusted to 10^6 protoplasts/ml.

Plant tissues such as leaf tip, leaf base, coleoptile and root tips were excised from seven-day-old seedlings of Triticale cv AC Alta grown on moistened-cotton-lined Petri plates in a growth room maintained at 22 °C with an 18 h light and 6 h dark cycle.

Onion epidermal cell layer was peeled fresh from onions.

Peptides Synthesis and Fluorophore Labeling

*p*VEC (LLIILRRRIRKQAHAHSK), scrambled *p*VEC (IAARIKL RSRQHIKLRHL) and transportan (GWTLNSAGYLLGKINLKA-LAALAKKIL) were custom-synthesized at the Alberta Peptide Institute, University of Alberta, Canada. Peptides were synthesized by solid-phase synthesis (model 430A, Applied Biosystems, Canada) using t-Boc strategy as reported by Hodges *et al.* (1988) [20]. For fluorophore labeling, carboxyfluorescein was added to the *N*-terminus of protected peptide before cleavage. Peptide cleavage from the resin was carried out with hydrofluoric acid. The product was purified on a Zorbax 300SB-C8 reverse phase column. Fractions were identified by analytical HPLC (HP1100) on a Discovery C8 column. Pure fractions were pooled and freeze-dried. The purified product was hydrolyzed with HCl and analyzed on a Beckman 6300 amino acid analyzer using System Gold. Peptide molecular weight was confirmed using MALDI-TOF (Voyager DE Pro) mass spectrometry.

Treatment of Various Plant Cells with Fluorescently Labeled CPPs

Plant cells were treated with 5 μM fluorescently labeled *p*VEC, transportan or scrambled *p*VEC for 1 h in dark at RT followed by washings and trypsin treatment. The steps associated with peptide treatment of the mesophyll protoplasts as described previously [14] were further extended to the other plant tissues investigated.

Fluorescence Microscopy and Fluorimetric Analysis

Plant cells were observed under the fluorescence microscope (Ex 490 nm/Em 520 nm) for cellular internalization of *p*VEC and transportan (for mesophyll protoplasts, a Nikon C1+ confocal Nikon Eclipse TE2000U microscope with epifluorescence was used; for onion epidermal cell layer, Olympus, BX51; for tissues, Leica MZ FLIII). Further studies employed protoplasts as the system of investigation owing to their abundance and ease of isolation and purification.

Fluorimetric analysis for the peptide treatments of mesophyll protoplasts was carried out using a fluorimeter (Biorad, Versafluor; Ex 490 nm/Em 520 nm). Protoplasts were lyzed with 400 μl of 1% TritonX-100 prepared in CPW for 20 min at 4 °C. FITC-dextran sulfate (MW 4 kDa, Sigma Aldrich) along with scrambled *p*VEC served as negative control in the preliminary experiments.

Effect of Different Factors and Inhibitors

Concentration. Protoplasts were treated with a range of concentrations of fluorescently labeled *p*VEC, transportan and scrambled *p*VEC (0–30 μM) following the same procedure as mentioned above.

Temperature. Protoplasts were preincubated at the required temperature (4–37 °C) for 20 min followed by addition of 5 μM fluorescently labeled *p*VEC, transportan and scrambled *p*VEC, and further incubated at the respective temperature for 1 h in dark.

Endocytic/Macropinocytic Inhibitors

Protoplasts were preincubated with the inhibitors for endocytosis (10 μM nocadazole, 5 mM sodium azide) and macropinocytosis (10 μM cytochalasin D, 100 μM EIPA) for 30 min followed by addition of 5 μM fluorescently labeled *p*VEC, transportan and scrambled *p*VEC for 1 h in dark at room temperature.

Three sets of experiments were carried for each treatment, and the average relative fluorescence uptake corresponding for each CPP was plotted.

RESULTS AND DISCUSSION

In the present study we investigated the ability of fluorescently labeled *p*VEC and transportan to translocate across various plant cells. Mesophyll protoplasts were chosen for further investigations to study the effect of factors influencing the cellular internalization of these CPPs.

We studied the uptake of *p*VEC and transportan in plants ranging from the single, wall-less plant cell (protoplast) to complex, differentiated plant tissues. We observed distinct translocation of the peptides in protoplasts (Figure 1(A) a–d) and onion epidermal cells (Figure 1(B) a–d). Among the various plant tissues, *p*VEC and transportan showed significantly weak fluorescence in leaf tips followed by coleoptile excised from seven-day-old triticale seedlings (not shown in the figure). However, leaf bases that are highly meristematic in nature and the root tips known for high mitotic activity showed significant internalization of both *p*VEC and transportan as observed by visual fluorescence microscopy (Figure 1(C) and (D) a–d). The controls without any treatment with the peptide did not show any fluorescence. Scrambled *p*VEC serving as another negative control showed significantly weak fluorescence (Figure 1). The present study corroborates that the extent of internalization of CPPs varies not only with the mammalian cell lines and different tissues but also the organisms [21–24].

Fluorimetric analysis of the cellular uptake of CPPs in protoplasts showed that the uptake of transportan was approximately 2.3 times higher than that of *p*VEC (Figure 2(A)). Under the fluorescence microscope, 25% of the protoplasts showed uptake of *p*VEC and 40% exhibited uptake of transportan, whereas

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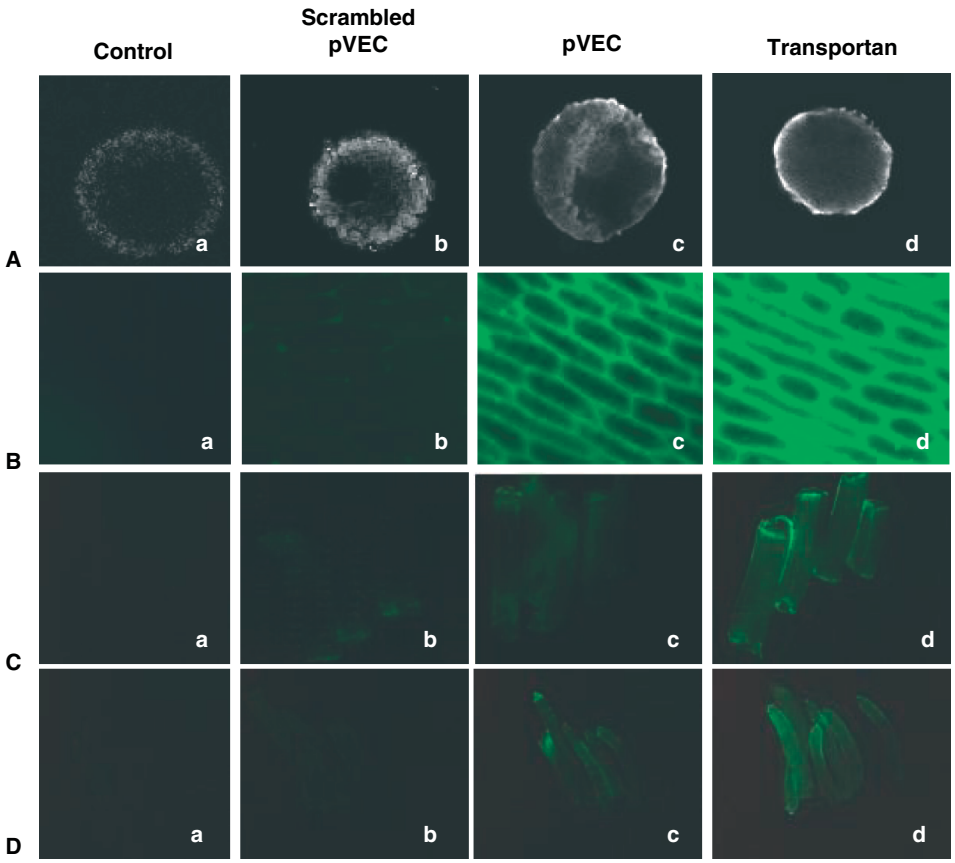

Figure 1 Internalization of *p*VEC and transportan in various plant cells by visual fluorescence microscopy. The cells were treated with 5 μM of fluorescent peptide for 1 h at room temperature: (A) Triticale cv AC Alta mesophyll protoplasts; (B) Onion epidermal cells; (C) and (D) Leaf bases and root tips, respectively, excised from seven-day-old seedlings of Triticale cv AC Alta. Two negative controls were included (a) and (b) Control (no peptide treatment) and fluorescently labeled scrambled peptide respectively (c) and (d) show treatment with fluoresecently labeled *p*VEC and transportan.

scrambled *p*VEC and dextran sulfate (nonpeptidic control employed in the initial experiments) showed negligible florescent protoplasts.

Being single celled and without cell wall, protoplasts offer a system comparable to mammalian cell lines. They can also be easily isolated and purified in large numbers. Therefore, in the present investigation mesophyll protoplasts were employed to study the effects of various factors on cellular internalization of *p*VEC and transportan.

Increase in the concentration of *p*VEC and transportan enhanced fluorescence in protoplasts (Figure 2(B)), strongly suggesting cellular uptake of CPPs is concentration dependent and nonsaturable in plant cells. Internalization of histones in petunia protoplasts has also been reported to be concentration dependent [25]. Cellular uptake of *p*VEC and transportan is also concentration dependent in yeast and Bowes melanoma cells, respectively [19,24].

At low temperature (4 °C), we noted an increase in the uptake of *p*VEC and transportan (Figure 3(A)). Higher uptake of *p*VEC (1.3 times) and transportan (1.5 times) at low temperatures indicates that the CPP

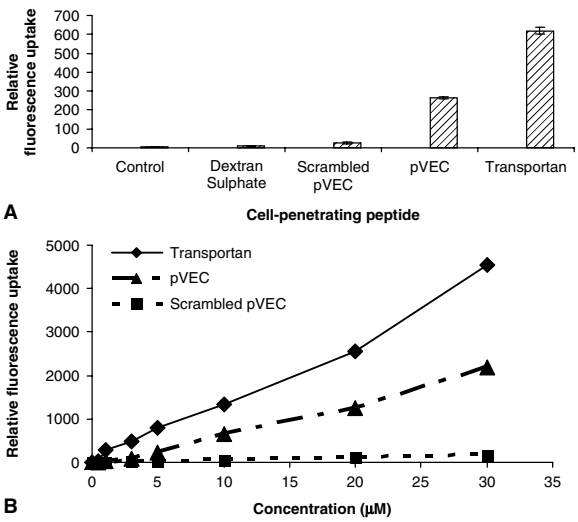

Figure 2 (A) Relative fluorescence uptakes of various fluorescently labeled CPPs in Triticale mesophyll protoplasts. The protoplasts were treated with 5 μM of fluorescent peptide for 1 h at room temperature. (B) Effect of concentration range on cellular uptake of *p*VEC, transportan and scrambled *p*VEC in triticale mesophyll protoplasts.

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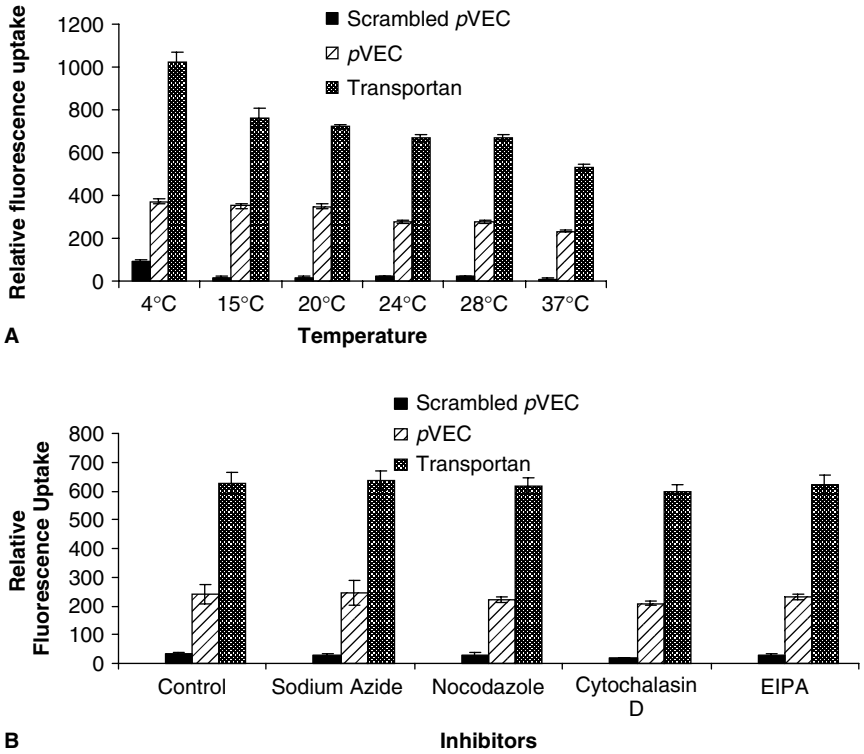

Figure 3 (A) Effect of temperature and (B) effect of endocytic/macropinocytic inhibitors on the cellular uptake of *p*VEC, transportan and scrambled *p*VEC in triticale mesophyll protoplasts.

entry into the protoplasts does not involve endocytosis but direct transduction across the cell membrane. Similar results were also observed with the histone uptake on petunia protoplasts [25]. This may be due to the low-temperature-induced change in the physicochemical properties of cell membrane favoring higher cellular uptake of the peptides studied [25–27]. FDA staining test for protoplast viability revealed that viability (70–80%) was not significantly compromised at the concentration of CPP used and there was no significant deleterious effect of the nonfluorescent transportan treatment on the protoplast viability (65%). However, protoplasts lose their integrity at higher temperature treatments (such as 37°C), resulting in lower fluorescence uptake (Figure 3(A)).

We observed that the pretreatment of the protoplasts with endocytic inhibitors such as nocadazole and sodium azide does not hamper the cellular uptake of *p*VEC and transportan in protoplasts. In the light of some recent reports suggesting the role of macropinocytosis in the cellular uptake of CPP–cargo complexes [10], we added commonly used macropinocytic inhibitors (cytochalasin D and EIPA) to the protoplasts to be treated with the labeled peptides. We did not find any significant effect of macropinocytosis inhibitors on the uptake of *p*VEC and transportan in mesophyll protoplasts (Figure 3(B)). However, noncovalent protein transduction of fluorescent proteins using arginine-rich CPPs in plant root tip cells is inhibited in the presence of the same macropinocytic inhibitors [13]. On the other hand, Elmquist and coworkers (2006) [28], reported that macropinocytic inhibitor EIPA has no significant effect on the uptake of *p*VEC in human Bowes melanoma cells. It is possible that the permeation properties of CPP change upon complexing with macromolecules, and as a result cargo is internalized by an altogether different mechanism(s) (which may involve macropinocytosis), whereas CPPs alone are transduced directly into the cells in an energy-, temperature- and receptor-independent manner.

We also noted that the uptake of scrambled *p*VEC was not significantly influenced by the various factors investigated, confirming that peptide sequence plays an important role in cell permeation properties of CPPs.

In our previous studies with fluorescently labeled, arginine-rich Tat peptides, the fluorescence distinctly accumulated in the nuclei of mesophyll protoplasts. In the present study, however, *p*VEC and transportan largely showed peripheral cytoplasmic fluorescence; in very few instances we observed the fluorescence outlining the nucleus in protoplasts treated with labeled transportan. It is interesting, as well as intriguing, that Tat, *p*VEC and transportan have very different amino acid compositions, but still these peptides show significant similarity in their uptake pattern under the influence of different factors in mesophyll protoplasts. On the basis of these observations, future studies may involve studying the CPP–plant membrane interaction in detail.

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CONCLUSIONS

Cellular internalization of fluorescently labeled *p*VEC and transportan was found to be tissue dependent in plants. Transportan showed higher fluorescence than *p*VEC, whereas scrambled *p*VEC showed weak fluorescence in all the plant cells tested. Mesophyll protoplasts being single-celled and devoid of cell wall served as an excellent system to further study the uptake of *p*VEC and transportan in plant cells. As observed in mammalian cell lines, our studies in plant protoplasts also indicate that the uptake of *p*VEC and transportan is concentration dependent and nonsaturable and does not involve endocytosis. The dynamic interaction of CPPs with cell membranes and their characteristic ability to deliver macromolecules that are much larger than their own size have the potential to open new research avenues in plant cell biology and biotechnology.

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