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Internalisation of cell-penetrating peptides into tobacco protoplasts

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Abstract

Cells are protected from the surrounding environment by plasma membrane which is impenetrable for most hydrophilic molecules. In the last 10 years cell-penetrating peptides (CPPs) have been discovered and developed. CPPs enter mammalian cells and carry cargo molecules over the plasma membrane with a molecular weight several times their own. Known transformation methods for plant cells have relatively low efficiency and require improvement. The possibility to use CPPs as potential delivery vectors for internalisation in plant cells has been studied in the present work. We analyse and compare the uptake of the fluorescein-labeled CPPs, transportan, TP10, penetratin and *p*VEC in Bowes human melanoma cells and *Nicotiana tabacum* cultivar (cv.) SR-1 protoplasts (plant cells without cell wall). We study the internalisation efficiency of CPPs with fluorescence microscopy, spectrofluorometry and fluorescence-activated cell sorter (FACS). All methods indicate, for the first time, that these CPPs can internalise into *N. tabacum* cv. SR-1 protoplasts. Transportan has the highest uptake efficacy among the studied peptides, both in mammalian cells and plant protoplast. The internalisation of CPPs by plant protoplasts may open up a new effective method for transfection in plants.

Keywords: Cell-penetrating peptide; Plant protoplast; Membrane penetration; Transportan; TP10; Penetratin; *p*VEC

1. Introduction

A new class of peptides was introduced ten years ago—cell-penetrating peptides (CPPs) by the discovery of the penetratin [1]. By definition, CPPs consist of less than 30 amino acids and have a net positive charge [2]. CPPs internalise in living animal cells in vitro [3,4] and in vivo [5,6] in a seemingly receptor- or energy- independent manner [7,8] with subsequent re-evaluation of the translocation mechanisms for some CPPs [9–11]. There are several classes of CPPs with various origins, from totally protein-derived CPPs via chimeric CPPs to completely synthetic CPPs. The mechanism of internalisation of CPPs is not yet characterised in detail, but the uptake seems to be different between the classes with a different element of endocytosis [12,13].

The plasma membrane is impenetrable for most polar hydrophilic macromolecules as proteins and oligonucleotides. Those molecules internalise into animal cells mainly via endocytosis. Several endocytotic mechanisms have been described [14] among which the clathrin-dependent receptor-mediated endocytosis is used for the internalisation of ligand–receptor complexes [15]. Most common procedures to deliver polar hydrophilic macromolecules into cells are electroporation and microinjection, but unfortunately the molecules can be delivered only in in vitro systems by these methods [16,17].

Penetratin, a 16-amino acid long peptide from the third helix of the *Drosophila melanogaster* Antennapedia homeodomain (amino acids 43–58) [1], was the first presented CPP, followed by many others like transportan, TP10 and

Abbreviations: CPP, cell-penetrating peptide; cv, cultivar; EDTA, ethylenediaminetetraacetate; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; Fl, fluoresceinyl; HEPES, *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HKR, HEPES-buffered Krebs-Ringer solution; RP-HPLC, reversed phase high performance liquid chromatography; RT, room temperature; *t*-Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; TP10, transportan 10

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Table 1
Cell-penetrating peptides used in this study

CPP	Sequence	Reference
Transportan	GWTLNSAGYLLGK(Fl)INLKALAALAKKIL-amide	[4]
TP10	AGYLLGKINLKALAALAKKIL-K(Fl)-amide	[18]
Penetratin	Fl-RQIKIWFQNRRMKWKK-amide	[17]
*p*VEC	Fl-LLIILRRRIRKQAHAHSK-amide	[19]
*p*VEC-scrambled	Fl-IAARIKLRSRQHIKLRHL-amide	[20]

*p*VEC. Transportan is a 27-amino acid long chimeric peptide including 12 amino acids from the neuropeptide galanin in the N-terminus connected with Lys13 to 14 amino acids from the wasp venom mastoparan in the C-terminus [5] (Table 1). Several transportan analogues have been synthesized [18], among these the shortest chimeric peptide that translocates through plasma membrane is transportan 10 (TP10). TP10 is truncated transportan analogue where six of the N-terminal amino acids have been deleted. *p*VEC is an 18-amino acid long peptide derived from murine vascular endothelial cadherin (amino acid 615–632) [19,20].

As the efficiency of different plant cell transfection methods is relatively low [21], it is desired to find a new better transduction method. CPPs are able to transport different macromolecules across animal cell plasma membrane—oligonucleotides [22], proteins [4], liposomes [23], peptide nucleic acids [24], nanoparticles [25], plasmids [26,27], siRNAs [28] and adenoviruses [29]. This gives hope that CPPs can be used as delivery vectors for macromolecules into plant cells. Here we test the ability of the fluorescently labeled CPPs, penetratin, transportan, TP10 and *p*VEC, to translocate into plant protoplasts, i.e. plant cells without cell wall.

2. Materials and methods

2.1. Peptide synthesis

Peptides (Table 1) were synthesized automatically (model 431A; Applied Biosystems) by solid phase methods using small scale (0.1 mmol) *t*-Boc strategy on *p*-methylbenzhydrylamin resin (Neosystem) (substitution 1.16 mmol/g) generating C-terminally amidated peptides. Stepwise coupling reactions were performed with *t*-Boc-protected amino acids (Neosystem), 1-hydroxybenzotriazole, *N*,*N*′-dicyclohexylcarbodiimide (4:4:4 eq, 35 min, RT) followed by N-terminal deprotection of the *t*-Boc-group with TFA:dichloromethane (1:1) (14 min, RT).

2.2. Fluorophore labeling of peptides

The peptides (for *p*VEC and penetratin at the N-terminal amino group, for transportan and TP10 at the side-chain of Lys^{13} and Lys^{22}, respectively) were coupled with 5(6)-carboxyfluorescein succinimidyl ester (Molecular Probes) (2 eq) and diisopropylethylamine (4 eq) in dimethyl sulfoxide:dimethylformamide (1:1) in dark overnight.

2.3. Deprotection and cleavage of peptides

Deprotection of the dinitrophenyl group was performed by treating the peptides (*p*VEC and *p*VEC-scrambled) with thiophenol:dimethylformamide (1:4) (1 h, RT) and the formyl group was removed by treating the peptides (transportan and penetratin) with piperidin:dimethylformamide (1:4) (30 min, RT). The final cleavage of the peptides from the resin was performed in hydrofluoric acid (1 h, 0 °C) in the presence of *p*-cresol and *p*-thiocresol (for *p*VEC and *p*VEC-scrambled only *p*-cresol was used).

2.4. Purification and characterisation of peptides

The peptides were purified by RP-HPLC (Discovery C-18 HPLC column, 25 cm, 21.2 mm, 5 μM) using a gradient of acetonitrile/water with 0.1% TFA (50 min, 8 ml/min). The identity and quality of the purified products were verified by matrix assisted laser desorption ionization time-of-flight mass-spectrometer (Voyager-DE STR, Applied Biosystems). The mass-spectra were acquired in positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix (Sigma-Aldrich) (10 mg/ml, 7:3 acetonitrile:-water, 0.1% TFA).

2.5. Cell culture

Bowes human melanoma cells (American Type Culture Collection CRL-9607) were cultivated in minimal essential medium with Glutamax I (Invitrogen), supplemented with 10% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids and 1% sodium pyruvate. Cells were incubated at 37 °C in 5% CO_2.

2.6. Protoplast preparation

Nicotiana tabacum cv. SR-1 plants were grown at 25 °C with a photoperiod of 16 h light and 8 h dark. For the protoplast preparation approximately 6-week-old plants were used. The enzyme solution containing 2.5% cellulase (Serva), 2.2% macerase (Novagen), and 20 ml buffer 1 (0.38% Gambourg's B5 salts (Duchefa), 500 mM mannitol (Duchefa), 2% sucrose (Sigma-Aldrich), 0.5% 4-morpholinethanesulfonic acid (Sigma-Aldrich), pH 5.7) was used for

the isolation of protoplasts. Two top leaves of *N. tabacum* cv. SR-1 were sterilised using 70% ethanol and 3% chloramine-T (Sigma-Aldrich) solution. The leaves were placed onto a Petri dish and buffer 1 solution (10 ml) was added. The leaves were cut into 1 mm pieces and more buffer 1 (10 ml) was added. The preparation was incubated in the dark (30 min) and then buffer 1 was replaced with enzyme solution, followed by incubation in the dark (16 h). The mixture of enzyme solution and protoplasts were poured through the sieve (50 mesh), centrifuged (15 min at 50×*g*, RT) and the supernatant was removed. 50% Percoll (Sigma-Aldrich) solution in buffer 1 (3 ml) was added to the pellet. This solution was placed into a 15 ml tube under the solution of 20% Percoll in buffer 1 and centrifuged (15 min at 200×*g*, RT). Intact protoplasts were transferred into a new tube, followed by the addition of buffer 2 (10 ml) (150 mM NaCl, 4 mM $CaCl_2$, 10 mM HEPES, 2% sucrose, 400 mM mannitol, pH 7.2) and centrifugation (15 min at 50×*g*, RT). The supernatant was removed and buffer 2 was added to get final protoplast concentration of 1 250 000 cells/ml.

2.7. Fluorescence microscopy

2.7.1. Bowes human melanoma cell line

The cells were seeded onto cover slides in a 24-well-plate (100 000 cells/well). One day after seeding semiconfluent (70–80%) cells were washed (3×1 ml HEPES-buffered Krebs-Ringer solution (HKR) (115 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 13.8 mM glucose, 20 mM HEPES, pH 7.4)) and the peptides were added (5 μM, 400 μl). As a negative control the FITC-labeled 4.4 kDa dextran (Sigma-Aldrich) was used. The cells were incubated with peptides (45 min, 37 °C) where after, the cells were washed (2×1 ml HKR) and treated with trypsin-EDTA (1:4):HKR solution (1:2) (4 min, 37 °C). The cells were washed (2×1 ml HKR) and analysed with microscope (Leica DMIRE2 fluorescence microscope, emission wavelength 520 nm).

2.7.2. N. tabacum cv. SR-1 protoplasts

On the day of the experiment the protoplasts (250 000) were exposed to a buffer 2 solution containing the peptide (5 μM, 400 μl). As a negative control the FITC-labeled 4.4 kDa dextran (Sigma-Aldrich) was used. The protoplasts were incubated with peptides (60 min, RT), then washed (2×1 ml buffer 2) and treated with trypsin-EDTA (1:4):buffer 2 solution (1:2) (4 min, RT). Protoplasts were washed (2×1 ml buffer 2) and analysed with a microscope (Olympus IMT2 fluorescence microscope, equipped with XF-100 filter).

2.8. Quantitative uptake estimation of peptides by spectrofluorometry

2.8.1. Bowes human melanoma cell line

The cells were seeded onto a 12-well-plate (200 000 cells/well). One day after seeding semiconfluent (70–80%) cells were washed (3×1 ml HKR) and the peptides were added (5 μM, 400 μl). As a negative control the FITC-labeled 4.4 kDa dextran was used. The cells were incubated with peptides (30 min, 37 °C), washed (2×1 ml HKR) and treated with trypsin-EDTA (1:4):HKR solution (1:2) (4 min, 37 °C). Cells were washed (2×1 ml HKR) and were lysed with 0.1% Triton X-100 in HKR (15 min, 0 °C), transferred into a 96-well plate and analysed with the SPEKTRAmax® GEMINI XS spectrofluorometric plate reader (Molecular Devices) at excitation wavelength 492 nm and emission wavelength 518 nm.

2.8.2. N. tabacum cv. SR-1 protoplasts

The protoplasts (250 000) were exposed to a buffer 2 solution containing the peptide (5 μM, 400 μl). As a negative control the FITC-labeled 4.4 kDa dextran was used. The protoplasts were incubated with peptide (60 min, RT), where after the protoplasts were washed (2×1 ml buffer 2) and treated with trypsin-EDTA (1:4):buffer 2 solution (1:2) (4 min, RT). Protoplasts were washed (2×1 ml buffer 2), lysed with 0.1% Triton X-100 in buffer 2 (15 min, 0 °C), transferred to a 96-well plate and analysed with the SPEKTRAmax® GEMINI XS spectrofluorometric plate reader (Molecular Devices) at excitation wavelength 492 nm and emission wavelength 518 nm.

2.9. Quantitative uptake estimation by FACS

2.9.1. Bowes human melanoma cell line

The procedure is the same as in Section 2.7.1, but after trypsin-EDTA treatment the cells were incubated with propidium iodide solution (10 μg/ml, 10 min). As negative controls fluoresceinyl-*p*VEC-scrambled and FITC-labeled dextran were used. The fluorescence was quantified with FACSort (FL1 and FL2) (BD Biosciences) and the results were analysed with CellQuest software (BD Biosciences).

2.9.2. N. tabacum cv. SR-1 protoplasts

The procedure is the same as in Section 2.7.2, but after trypsin-EDTA treatment the cells were incubated with propidium iodide solution (10 μg/ml, 10 min). As negative controls fluoresceinyl-*p*VEC-scrambled and FITC-labeled dextran were used. The fluorescence was quantified with FACSort (FL1 and FL2) (BD Biosciences) and results were analysed with CellQuest software (BD Biosciences).

3. Results

The ability of fluorescein-labeled peptides to internalise into *N. tabacum* cv. SR-1 protoplasts was determined with three different methods: fluorescence microscopy, spectrofluorometry and FACS analysis. Each method was performed both on mammalian cells and plant protoplasts.

3.1. Analysis of the internalisation of CPPs into mammalian and plant cells using fluorescence microscopy

Fluoresceinyl-transportan, -TP10 and -penetratin entered Bowes human melanoma cells at 37 °C (Fig. 1). The internalisation of 5 μM fluorescein-labeled peptides into living Bowes cells were assessed after 45 min incubation at 37 °C. No uptake was detected for the negative control, FITC-dextran, which indicate an intact plasma membrane (Fig. 1).

Thereafter, the ability of CPPs to penetrate into *N. tabacum* cv. SR-1 protoplasts was investigated. Tobacco protoplasts were incubated with fluorescein-labeled CPPs and the fluorescence was detected inside protoplasts (Fig. 2) (data shown for transportan). No uptake was detected for the negative control, FITC-labeled dextran (Fig. 2).

3.2. Analysis of the internalisation of CPPs into mammalian and plant cells using spectrofluorometry

100% of fluorescence indicates the total amount of fluorescence of added peptides. The results in Fig. 3A show that the best penetration efficiency into Bowes human melanoma cells has the fluoresceinyl-transportan (13% of added peptide) then fluoresceinyl-penetratin (2.8% of added peptide) and the lowest efficiency has fluoresceinyl-TP10 (2.2% of added peptide). With the experiments of plant protoplasts the results were as follows: fluoresceinyl-transportan (13.8% of added peptide), fluoresceinyl-TP10 (4.9% of added peptide) and fluoresceinyl-penetratin (2.2% of added peptide) (Fig. 3B).

3.3. Analysis of the internalisation of CPPs into mammalian and plant cells using FACS

Distinct from other methods used in this work it is possible to isolate different cell populations with flow cytometry. In order to isolate cells with intact membranes from the cells with permeabilised membranes, the cells were incubated with propidium iodide solution (10 μg/ml) 10 min before analysis.

100% indicates the total amount of investigated cells. From the whole population of Bowes human melanoma cells with intact membrane, 90.7% was internalised with fluoresceinyl-transportan, 97.1% with fluoresceinyl-TP10, 95.9% with fluoresceinyl-penetratin and 92.7% with fluo-

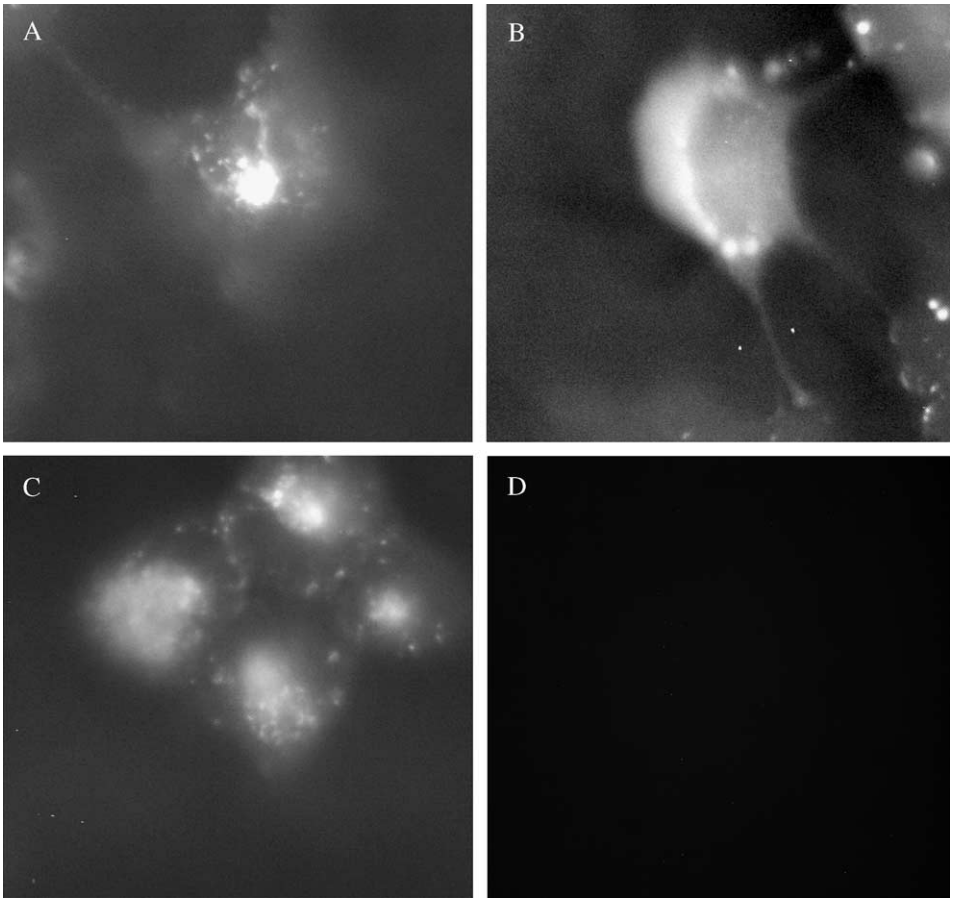

Fig. 1. Internalisation of fluorescein-labeled CPPs into Bowes human melanoma cells, visualised by fluorescence microscopy. The cells were incubated with 5 μM fluoresceinyl-CPPs in HKR solution for 45 min at 37 °C. (A) Fluoresceinyl-transportan; (B) fluoresceinyl-TP10; (C) fluoresceinyl-penetratin; (D) FITC-labeled dextran as a negative control.

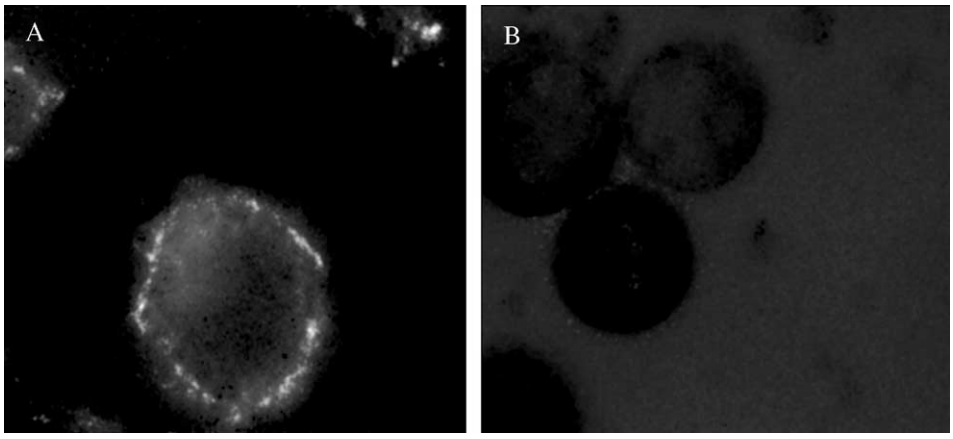

Fig. 2. Internalisation of fluorescein-labeled transportan into *N. tabacum* cv. SR-1 protoplasts, visualised by fluorescence microscopy. The cells were incubated with 5 μM fluoresceinyl-transportan in buffer 2 for 60 min at RT. (A) Fluoresceinyl-transportan; (B) FITC-labeled dextran as a negative control.

resceinyl-*p*VEC (Fig. 3C). As negative controls, FITC-labeled dextran and fluoresceinyl-*p*VEC scrambled were used, the internalisation was in the range of 0.06% and 1.68% (data not shown), respectively.

From the *N. tabacum* cv. SR-1 protoplast population the results were as follows: From 100% of cells with intact membrane, 15.7% were internalised with fluoresceinyl-transportan, 10.6% with fluoresceinyl-TP10, 2.8% with fluoresceinyl-penetratin and 5.9% with fluoresceinyl-*p*VEC (Fig. 3D). As negative controls, FITC-labeled dextran and fluoresceinyl-*p*VEC scrambled were used, the internalisation was 0.07% and 0.01% (data not shown), respectively.

4. Discussion

Recent reports from the last decade demonstrate the CPP-facilitated uptake of different macromolecules into mammalian cells. This has not been shown for plant cells until now and here we characterise the internalisation parameters of CPPs in plant protoplasts.

Our experiments show that CPPs enter mammalian cells with almost 100% efficiency (Fig. 3C), the efficiency into plant protoplasts is, however, significantly lower (Fig. 3D). This could be explained by the difference between animal and plant cell plasma membrane which mostly is in the sterol

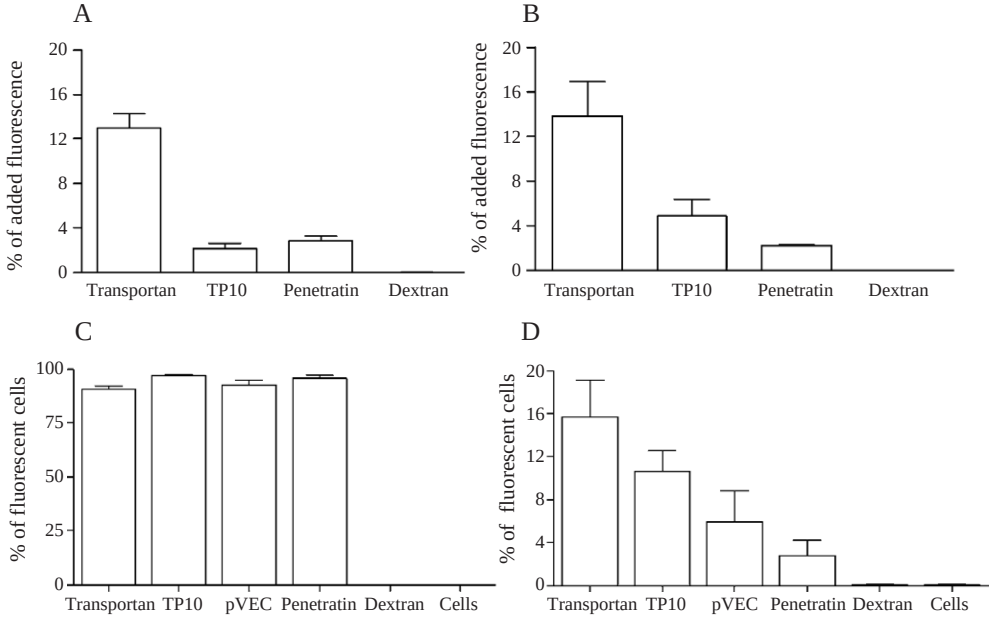

Fig. 3. Internalisation of fluorescein-labeled CPPs into Bowes human melanoma cells and *N. tabacum* cv. SR-1 protoplasts, detected spectrofluorometrically (A, B) and by FACS (C, D). (A) Bowes human melanoma cells were incubated with 5 μM fluoresceinyl-CPPs in HKR solution for 30 min at 37 °C. After the incubation diluted trypsin-EDTA:HKR (1:2) was added to remove the peptides on the cell membrane and then cells were lysed with Triton X-100. (B) *N. tabacum* cv. SR-1 protoplasts were incubated with 5 μM fluoresceinyl-CPPs in buffer 2 for 30 min at RT. After the incubation diluted trypsin-EDTA:buffer 2 (1:2) was added to remove the peptides on the cell membrane and then cells were lysed with Triton X-100. FITC-dextran was used as a negative control. The fluorescence was measured at 518 nm with a spectrofluorometric plate reader. The ordinate shows the quantity of added peptides fluorescence. (C, D) The Bowes human melanoma cells (C) and *N. tabacum* cv. SR-1 protoplasts (D) were incubated with 5 μM fluoresceinyl-CPPs for 60 min. The TP10 used in FACS studies labeled with fluorescein at Lys^7. The ordinate shows the quantity of the cells internalised with peptides.

composition. The major sterol in mammalian cells is cholesterol, while plant cell membranes contain other types of sterols like sitosterols, stigmasterols and campesterols [30]. Differences have also been found in glycerolipid fatty acid chains, stearic acids and arachidonic acids in mammalian cells versus linolenic and linoleic acids in plant cells. Sphingomyelin, a common mammalian cell plasma membrane component, has not been found in plant cells, however. For penetratin it has been shown that the presence of a transbilayer potential as well as the bilayer composition is important for the uptake [31].

Since now, the ability to penetrate into plant cells has been demonstrated only for core histones [32] (i.e. large proteins known to work in the nucleus where DNA is wrapped around octamers of histones). The histones are positively charged and DNA is electrostatically bound to the histones. It has been previously shown that core histones are able to cross mammalian cell plasma membranes and carry macromolecules covalently attached to them across the plasma membrane [33]. The core histones consist of 102–135 amino acids [34], which makes them considerably longer than the CPPs [2]. The possibility to use CPPs instead of histones to carry macromolecules across the plasma membrane would facilitate plant research and manipulation.

The efficiency of different available transformation methods for plant cells is low, not routinely performed, and most of them are not able to yield systemic transformation [21]. Therefore CPPs could have practical value as tools for translocating biomolecules into plant cells.

The CPPs used here are well characterised in mammalian cells [2]. The negative controls, fluoresceinyl-*p*VEC scramble and FITC-labeled dextran, were chosen because they lack cell penetration ability [20], and their molecular weights are similar to the CPPs utilized in this work. To remove membrane bound peptide the cells were treated with diluted trypsin before analysing the amount of intracellular peptide [35].

The internalisation of CPPs into plant protoplasts opens up a new and better method for genetic modification of plants. By coupling peptides, oligonucleotides, plasmids and peptide nucleic acids to CPPs it can be possible to effectively transport them inside the plant protoplasts.

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