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Gene Delivery to Tobacco Root Cells with Single-Walled Carbon Nanotubes and Cell-Penetrating Fusogenic Peptides

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Abstract

Development of efficient, easy, and safe gene delivery methods is of great interest in the field of plant biotechnology. Considering the limitations of the usual transfection methods (such as transgene size and plant type), several new techniques have been tested for replacement. The success of some biological and synthetic nanostructures such as cell-penetrating peptides and carbon nanotubes in transferring macromolecules (proteins and nucleic acids) into mammalian cells provoked us to assess the ability of an engineered chimeric peptide and also arginine functionalized single-walled carbon nanotube in gene delivery to intact tobacco (*Nicotiana tabacum* var. Virginia) root cells. It was suggested that the engineered peptide with its special cationic and hydrophobic domains and the arginine functionalized single-walled carbon nanotube due to its nano-cylindrical shape can pass plant cell barriers while plasmid DNA (which codes green fluorescent protein) has been condensed on them. The success of gene delivery to tobacco root cells was confirmed by fluorescence microscopy and western blotting analysis.

Keywords Engineered chimeric peptide · Functionalized carbon nanotube · Green fluorescent protein · Tobacco root · Transfection

Introduction

For several years, *Agrobacterium* and gene gun have been the most popular tools for plants transformation; however, their drawbacks such as being efficient only in special plant types and requiring expensive equipment restricted their use. In the last decade, several new methods have been investigated for previous ones replacement. The cell-penetrating peptides (CPPs) have been recently shown to efficiently deliver macromolecules into mammalian cell [1, 2]. In recent years, the ability of some CPPs in carrying macromolecules into plant cells has been studied. Several reports indicated that cationic (Tat, Tat2 and polyarginine) and hydrophobic (Pep-1) cell-penetrating peptides not only can pass through plant cell barriers (cell wall and plasma membrane) but also can transfer macromolecules such as protein and nucleic acids to the cells [3–5]. In addition to CPPs, core histones (H2A, H2B, H3, and H4) could successfully carry proteins to protoplasts and intact cells [6]. The success of synthetic chimeric peptides in delivery of the cargos to intact plant cell has been reported [7, 8]. A cell environment-responsive CPP was tested for more efficient delivery and release of the associated DNA cargo [9]. CPPs in conjugation with organelle-targeting peptides were used to deliver gene and proteins with a wide range of molecular weights to intact plant cells organelles [10, 11].

In addition to biological nanoparticles, some synthetic nanostructures such as carbon nanotubes (CNTs) have been used as new carriers to transfer macromolecules into living cells. The significant hydrophobicity of CNT, which results in weak water solubility and biocompatibility, can be decreased by surface modifications. CNT functionalization with positively charged compounds greatly improves the solubility and CNT–DNA interactions. Single-walled

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and multi-walled CNTs functionalized with ammonium [12] polyethylenimine [13, 14] and cationic amphiphile such as pyrenyl polyamine [15] have been used to deliver DNA and siRNA to mammalian cells. Some researches have confirmed carbon nanotubes internalization to the intact plant cells and the induction of growth enhancement and seed germination [16–18]. In 2009 for the first time, single-walled CNT has been reported to deliver a short single-stranded DNA (ssDNA) into intact tobacco cells [19]. Recently, transfection of plasmid DNA to mesophyll protoplasts and callus cells by carbon nanotubes has been reported [20].

In this research, an engineered fusion peptide, which has been demonstrated to be able to efficiently pass the plasma membrane [21] and arginine functionalized single-walled carbon nanotubes (Arg-SWNT), were investigated as gene delivery tools to tobacco root cells. The fusion peptide was composed of three distinct parts (Fig. 1) [22]. The truncated H1 (ATPKKSTKKTPKKAKKATPKKSTK-KTPKKAKK) was supposed to condense DNA intensely due to its abundant lysine residues while the conserved motif of HIVgp41 (GALFLGFLGAAGSTMGA) guarantees the endosomal escape by adopting α helical structure in acidic pH. The nuclear localization signal (NLS) (PKKKRKV) increases the possibility of cargo transfer to the nucleus. The conjugation of the conserved motif of HIVgp41 to nuclear localization signal has been known as MPG, which is a successful CPP in gene delivery to mammalian cells [23, 24]. It was suggested that the attachment of two repeats of truncated H1 could change the peptide to a more powerful CPP which could increase the transfection efficiency.

In addition, arginine functionalized single-walled carbon nanotubes were used to transfer plasmid DNA (encoding green fluorescent protein (GFP) gene) to tobacco intact root cells. The arginine functionalized CNT is water soluble and biocompatible [25] and is suggested to be able to interact with DNA and pass through cell wall pores and plasma membrane.

Materials and Methods

Plant Root Preparation

Sterilized tobacco seeds were soaked in water for about 20 h. The soaked seeds were placed on wet filter paper in a Petri dish. The 6-day-old seedlings were used for transfection.

Peptide Overexpression

MPG-2H1 has been cloned into pET28a (Novagen) expression vector via *Nde*I and *Xho*I restriction sites [21]. The transformed bacteria (*E. coli* C41(ED3)) were pre-cultured in LB medium in the presence of Kanamycin and incubated at 37 °C and 220 rpm shaking overnight. The bacteria were then transferred to 2XYT medium containing Kanamycin and incubated in the same condition until OD_{600} of the medium reached 0.4. The protein expression was induced by addition of IPTG to the final concentration of 0.2 mM and the culture was incubated in the previous condition for 4 h [21].

Peptide Extraction and Purification

The harvested bacteria were resuspended in lysis buffer [20 mM Tris, 500 mM NaCl, 8 M urea and 5 mM imidazole; (pH 11)] and incubated at room temperature for an hour. After centrifugation, the supernatant was loaded on Ni–NTA chromatography resin (Qiagen) and left for an hour at room temperature [21]. The resin was then washed with washing buffers containing increasing concentration of imidazole and decreasing concentration of urea [20 mM Tris, 1 M NaCl, 20 mM, 40 mM, 60 mM and 80 mM imidazole, 7 M, 6 M, 4 M, 2 M and 0 urea; (pH 8)] (according to majidi et al. [21] with some modifications). Finally, the peptide was eluted with elution buffer [20 mM Tris, 500 mM NaCl and 250 mM imidazole; (pH 8)]. The purified peptide was analyzed by 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Buffer exchange carried out by the use of dialysis tubing (2 kDa molecular weight
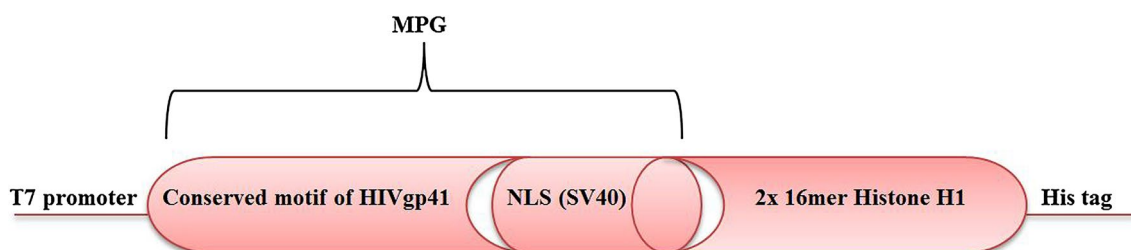

Fig. 1 Schematic representation of the chimeric peptide with three distinct parts. Conserved motif of HIVgp41: GALFLGFLGAAGSTMGA, NLS: PKKKRKV, 2×16mer Histone H1: ATPKKSTKKTPKKAKKATPKKSTKKTPKKAKK

cut-off, Sigma-Aldrich). Dialysis tubing was filled with purified peptide and immersed in phosphate buffered saline (PBS) for 48 h. PBS buffer was changed three times during this period.

Single-Walled Carbon Nanotubes Functionalization with Arginine

To functionalize CNTs with arginine, 80 mg raw CNTs (US Research Nanomaterials) were mixed with 40 ml mixture of concentrated sulfuric and nitric acids (3:1) and refluxed at 45 °C for 20 h. The SWNTs-COOH were then washed several times with distilled water and treated with 25 ml of thionyl chloride ($SOCl_2$) (Merck) and dimethylformamide (DMF) (Merck) mixture (24:1) and refluxed at 65 °C for 24 h. The excess $SOCl_2$ was removed by the use of rotary evaporator. The acylated CNTs (CNTs-COCl) were resuspended in 40 ml tetrahydrofuran (THF) (Merck) and mixed with 3 g L-arginine (Sigma) by probe sonicating for 50 min [26]. The mixture was refluxed for 5 days at room temperature. Finally tripotassium phosphate (K_3PO_4) (Merck) at the final concentration of 750 mM [27] was added and the mixture refluxed for 24 h (Fig. 2). Functionalized SWNTs harvested by centrifugation (16,000 rpm for 30 min) and resuspended in distilled water and lyophilized for long-term storage.

Fourier Transform Infrared Spectroscopy (FTIR)

A mixture of KBr and CNT samples was compressed to form disks and used for infrared spectroscopy. The spectra were recorded with FTIR spectrometer PerkinElmer (MA, USA) 2000; operating in the range of 400–4000 cm^{-1}.

Plasmid DNA Preparation

The pHBT-sGFP(S65T) in *E. coli* has been provided by ABRC (Arabidopsis Biological Resource Center). This plasmid 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 [28]. The plasmids were extracted by the use of plasmid extraction kit (GeneAll Company). The extracted plasmid has been analyzed on 1% agarose gel.

DNA–Peptide Complex Formation and Characterization

200 ng of extracted plasmids was mixed with different amounts of recombinant peptide to form complexes with different N/P ratios. This ratio is referring to the number of nitrogen residues (N) in the peptide per phosphate groups (P) of DNA. The mixtures were left for 30–45 min at room temperature and then were loaded on 1% Agarose gel to show the differences in electrophoretic mobility of the complexes [21].

Size and zeta potential of the DNA–peptide complexes, formed at different N/P ratios were measured by Zetasizer Nano ZS instrument (Malvern Instruments, UK).

SWNT–Plasmid Complex Formation and Characterization

200 ng of extracted plasmids was mixed with different amounts of functionalized carbon nanotubes. The CNT–plasmid mixtures were left at 4 °C for 24 h to form complexes [29]. The complexes were loaded on 1% agarose gel.
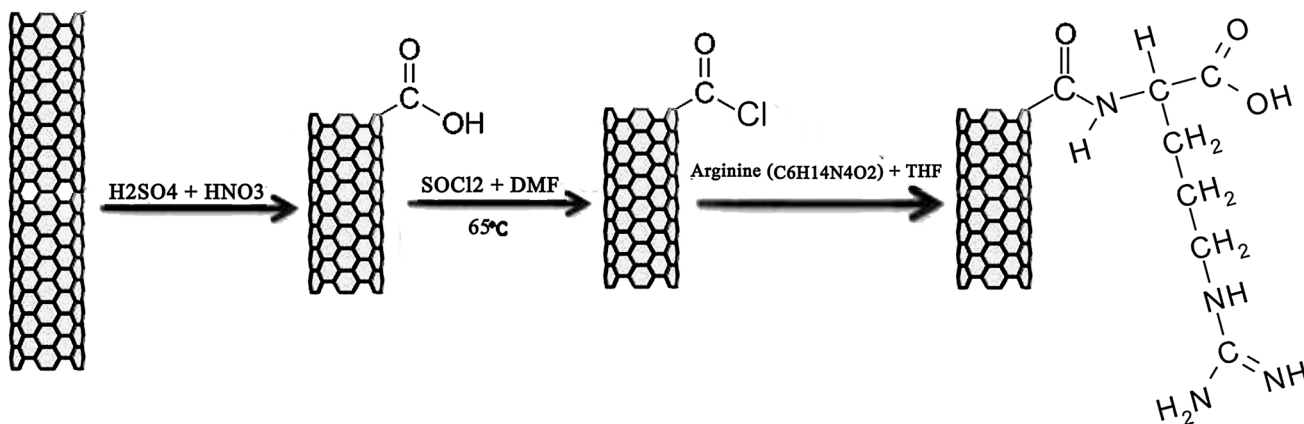

Fig. 2 Schematic representation of the carbon nanotube functionalization by arginine

Cell Wall Enzyme Treatment

The roots were treated by pectinase and cellulase (Sigma) to loosen cell wall pores. 1 g of 6-day-old seedlings was treated with pectinase solution (sucrose 0.5 mM, CaCl$_2$ 20 mM, KCl 40 mM, MES-KOH 20 mM (pH 5.7), Pectinase 1%) and gently shaked at room temperature for 8 h. Another enzyme solution with 1% pectinase and 1.5% cellulase was used to treat 1 g of seedlings for 4 h at room temperature with gentle shaking [30].

Nanostructure Preparation and Transfection

Peptide–plasmid complexes were added to 6-day-old roots separately and incubated for 4 h at 25 °C with gentle shaking (50 rpm). Thereafter, the transfection solution was diluted to half concentration and the roots were incubated in it for 20 h (Fig. 3). For Arg-SWNT–plasmid complex preparation 10 μg plasmid was mixed with 10, 80, and 200 μg functionalized carbon nanotube separately and left for 24 h at 4 °C. The prepared complexes were added to the roots followed by incubation at 25 °C and shaking at 50 rpm for 24 h (Fig. 4). The GFP expression by the root cells was investigated with

fluorescence microscopy using Cytation3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT), RT-PCR, and western blotting analyses.

Plant Protein Extraction

0.2–0.3 g transfected seedlings were grinded in liquid nitrogen by the use of mortar and pestle. The seedlings powder was mixed with 2 ml of TCA (10% in acetone) followed by centrifugation at 12,000 rpm, 4 °C for 3 min. The pellet was resuspended in acetone and again sedimented with centrifugation as described. This step was repeated until the color of the pellet turned white. In the next step, the pellet was washed with 0.1 M ammonium acetate in methanol, after centrifugation, the sediment was resuspended in phenol–SDS buffer (1:1) (SDS buffer: sucrose 700 mM, Tris 100 mM, 2β Mercaptoethanol 0.1% and SDS 1%). The lysed cells were centrifuged (12,000 rpm at 4 °C for 5 min) and the phenolic phase (the upper phase) was transferred to a new tube. The tube was filled with 0.1 M ammonium acetate in methanol and incubated at −20 °C overnight. The precipitated protein pellet was washed with methanol and acetone
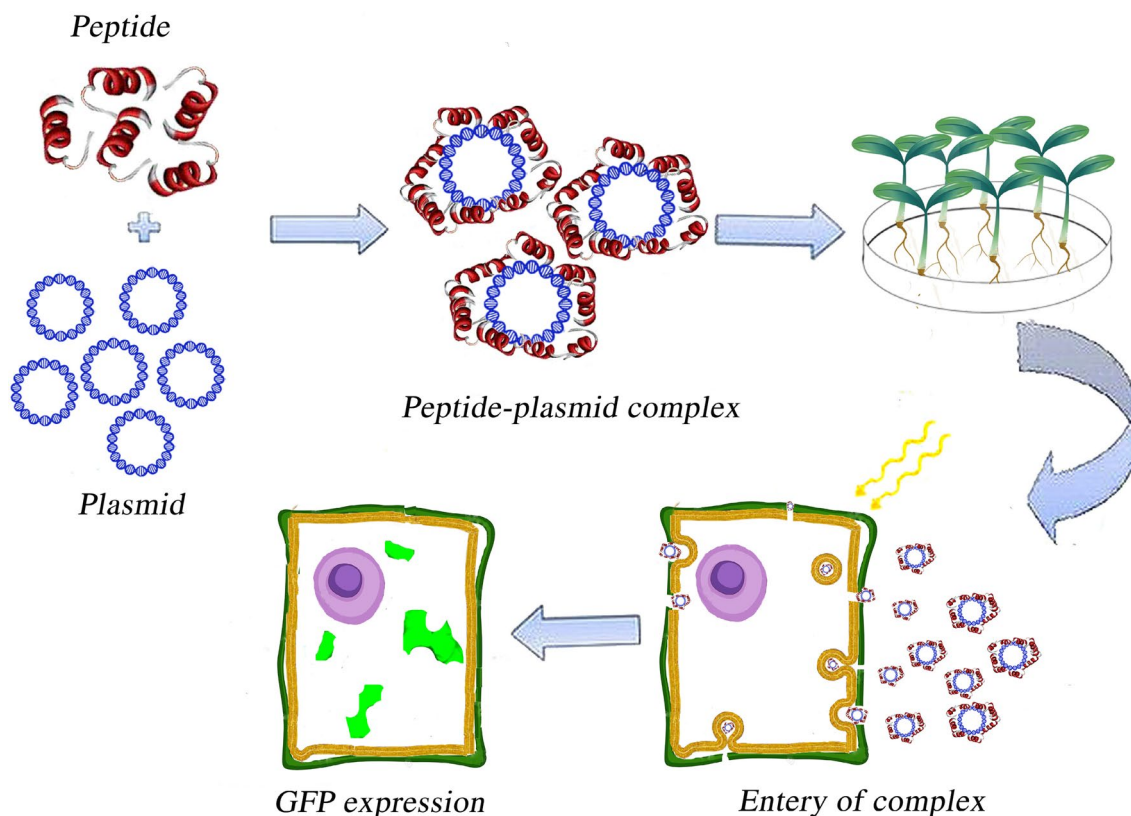

Fig. 3 Schematic representation of the root cell transfection with peptide–plasmid complexes. The complexes formation, internalization of the complexes into the cell, and GFP protein expression
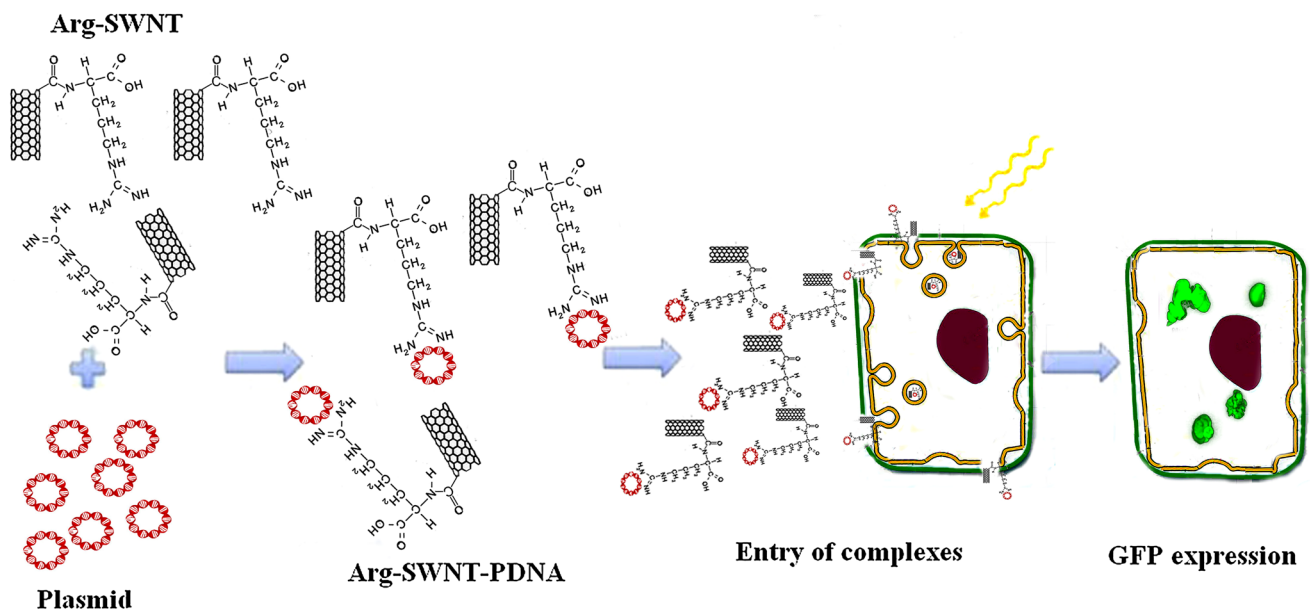
Fig. 4 Schematic representation of the root cell transfection with Arg-SWNT–plasmid complexes. The complexes formation, internalization of complexes, and GFP protein expression

80%, respectively, and resuspended in 20 µl PBS buffer containing 8 M of urea [31].

Enhanced Chemiluminescence (ECL) Western Blotting

Extracted protein from 0.3 g of transfected seedlings was electrophoresed on 12.5% gel and transferred to nitrocellulose membrane. Transferring process was conducted in transfer tank (Bio-Rad) using 100 mA constant electric current for 150 min. The membrane was then blocked by Tris-buffered saline, Tween 20 (0.1%), and BSA (1%) for 3.5 h. Anti-GFP antibody (Biolegend) as and HRP goat anti-rat antibody (Biolegend) were applied to detect GFP on the membrane. Finally, the paper was covered with chemiluminescent substrates (Luminol A and B) and the emitted light was recorded with ECL detector [Western Blot Imaging System (SABZ Biomedicals Company, Iran)] [32].

Results and Discussion

With the aim of developing safe and efficient plant transfection methods, several new carriers have been investigated so far. Cell-penetrating peptides and single-walled carbon nanotubes have attracted attention as they have demonstrated to be able to pass through the cell wall nano-pores and the plasma membrane. Cationic [33] and hydrophobic [34] CPPs have been used as tools for nonviral gene transfer to mammalian cells. Here in, a recombinant fusion peptide containing cationic and hydrophobic domains was exploited to deliver GFP coding plasmids to intact tobacco root cells. Arginine functionalized single-walled nanotubes were also used to carry the same plasmid to tobacco root cells. Carbon nanotubes can cross the cell membrane either by direct penetration or endocytosis [35, 36]. Arginine functionalization of carbon nanotubes increases solubility and also DNA adsorption by providing electrostatic interactions. It has been shown that polyarginine can act as a nuclear localization signal similar to SV40 NLS (PKKKRKV) [37] leading to the transfer of cargo to the nucleus.

Confirmation of Peptide–Plasmid Complex Formation

The results of gel retardation assay showed that even at low N/P ratios (N/P:0.5), plasmid has been trapped completely by the fusion peptides (Fig. 5). Both the positive charge and the size of the peptide–plasmid complexes account for the retardation of the complexes on the gel. The results demonstrated that this peptide is even more efficient in trapping of the plasmids than the previously reported peptides [7, 38].

As can be seen in Fig. 6, by increasing N/P ratios better condensation of the complexes occurs and the size of the

nanoparticles decreases. The size of the carrier has a direct impact on the efficiency of passage through the cell wall pores. Considering the negative net charge of the plasma membrane of the plant cells, nanoparticles with positive charge can efficiently interact with the cell membrane by electrostatic interactions. Nevertheless, the passage of negatively charged particles through the cell wall and cell membrane had been reported before [7].

Arginine Functionalization of SWNT

SWNT functionalization was verified with FTIR spectroscopy and zeta potential measurements. FTIR spectra illustrated a peak at ~ 1712 cm^{-1} which is related to stretching of carbonyl group in SWNT-COOH. This peak shifted to 1619 cm^{-1} in the spectrum of Arg-SWNT, which indicated that carboxylic groups of SWNT-COOH have reacted with the amino groups of arginine and turned into amide groups. The peak at 1612 cm^{-1} in

Fig. 5 Gel retardation assay of the peptide–plasmid complexes. From right to left: 200 ng plasmid, complexes with N/P: 0.5, 1, 2, 4, 6, 10, 14, and 18. The retardation of the plasmids on the gel indicates the peptide–plasmid complex formation
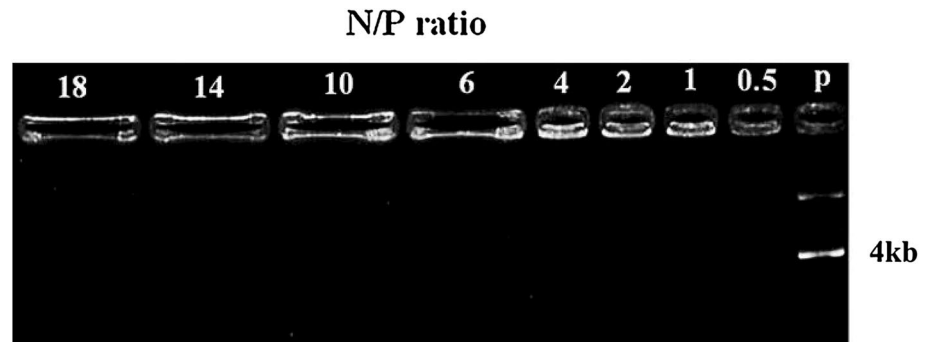

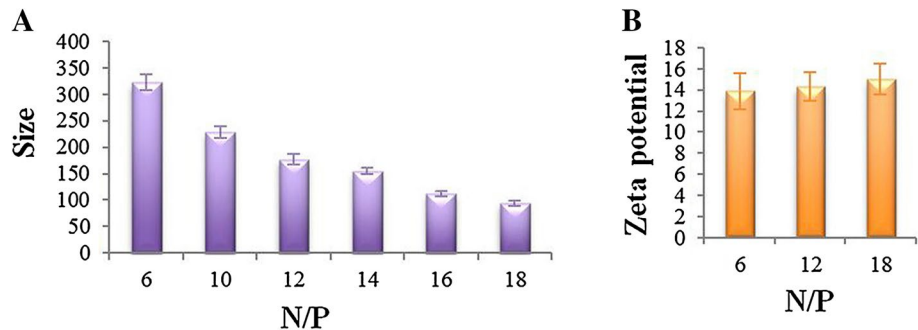**Fig. 6** The particle size (**A**) and zeta potential (**B**) of the peptide–plasmid complexes at different N/P ratios
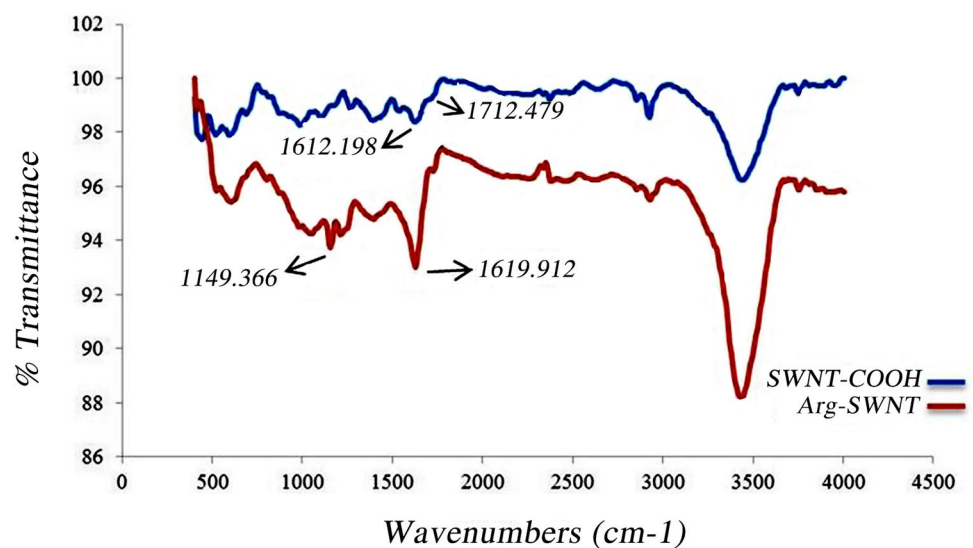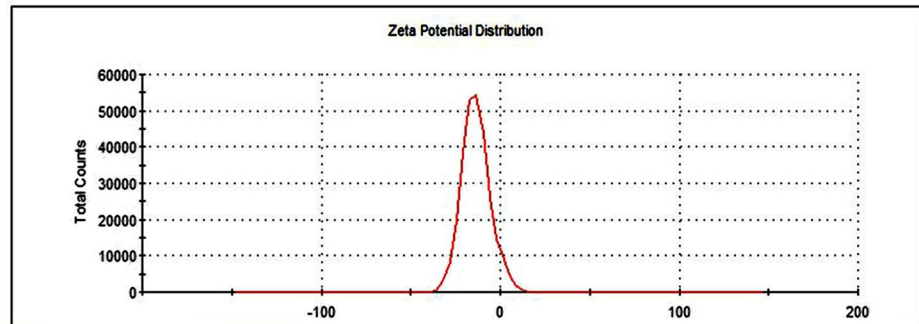

Fig. 7 FTIR spectra of SWNT-COOH and Arg-SWNT

Fig. 8 Zeta potential of pristine SWNT (**A**), SWNT-COOH (**B**), and Arg-SWNT (**C**)

SWNT-COOH can be assigned to stretching vibration of water [39]. In addition, the observation of C–N stretching band at 1149 cm^{-1} is referred to amide bond formation [40] (Fig. 7).

Since arginine is a positively charged amino acid at physiological pH, the arginine functionalized carbon nanotubes are expected to be less negatively charged compared to the SWNTs-COOH. The results showed that the zeta potential of SWNT-COOH has reduced from −42.1 to −27.2 mV after treating with arginine, confirming the success of the functionalization process (Fig. 8).

Gel Retardation Assay

The plasmids were mixed with different amounts of functionalized carbon nanotubes and the complexes were loaded on 1% agarose gel. Negatively charged Plasmids bind to positively charged arginine residues on the surface of SWNT by electrostatic interactions. The Arg-SWNT–plasmid complexes showed lower mobility on the gel compared to the naked plasmid (Fig. 9). The gel retardation assay confirmed the interaction between the plasmid and functionalized SWNT at all ratios of plasmid to carbon nanotube. The results demonstrated that by increasing the amount of CNT, the retardation of the plasmids increases. However, at lower ratios of plasmid to SWNT (1/10 and 1/20), less retardation of the plasmids was seen.
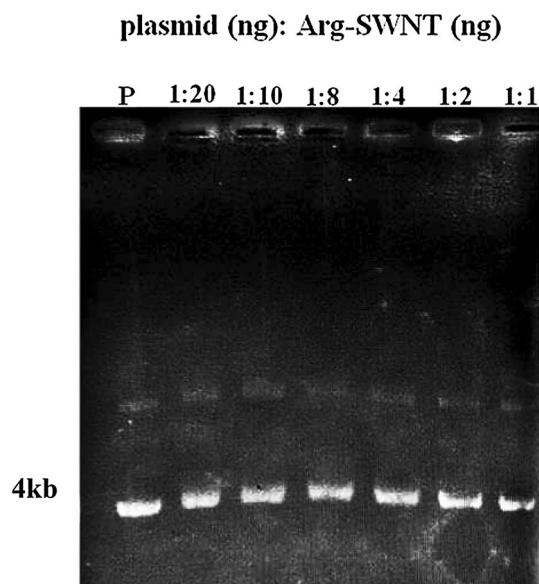
plasmid (ng): Arg-SWNT (ng)

Fig. 9 Gel retardation assay of Arg-SWNT–plasmid complexes. From left to right: 200 ng of plasmid, 200 ng of plasmids in complex with 4, 2, 1.6, 0.8, 0.4, and 0.2 µg of Arg-SWNT, respectively. The functionalized carbon nanotube could trap plasmid DNA even at low concentrations

This can be due to the carbon nanotubes aggregation at higher concentrations.

Transfection of Intact Tobacco Root Cells by Peptide–Plasmid Complexes

In spite of the fact that smaller particles have more chances for passing through the cell wall, the smallest peptide–plasmid complexes will not be necessarily the best carriers. Since the smaller complexes were packed intensely, the unpacking process inside the cells might encounter difficulties [41, 42]. In large complexes that are not packed very well, the plasmids are surrounded by fewer peptides. In these complexes not only the large size is a great obstacle to pass through the cell wall pores but also the presence of fewer α helices of HIVgp41 segments that are responsible for endosomal escape would be another drawback [43, 44]. To find the best molar ratio of peptide to plasmid, different N/P ratios had been prepared and their transfection efficiencies were compared by fluorescence microscopy imaging. Significant differences between transfected and control roots were observed. The results are in parallel with previous reported flourescence microscopy images of GFP gene delivery to plant cells by *Agrobacterium* [45], arginine-rich peptide [37], and polyethylene glycol [28]. Based on our observation, N/P = 6 and 12 resulted in satisfactory transfection efficiencies although in other N/P ratios some transfected root cells could be observed (Fig. 10a, b). Although in almost all of the previous reports the efficiency of transfection by means of the peptides has been proved only by fluorescence microscopy [7, 37, 46], we believe that the expression of the reporter gene needs to be further analyzed by RT-PCR and/or western blotting.

Transfection of Intact Tobacco Root Cells by Arg-SWNT–Plasmid Complexes

Different amounts of Arg-SWNT (10, 20, 40, 80, 250, and 400 µg) were used to transfer 10 µg of plasmids into tobacco root cells. Fluorescence microscopy images illustrated that all concentrations of functionalized CNT have successfully transfected tobacco root cells (Fig. 11a–c). Considerable reduction in transfection efficiency was observed at higher concentrations of SWNT, which can be due to the increased aggregation of carbon nanotubes.

Analysis of GFP Expression in Tobacco Root Cells Transfected by Peptide–Plasmid and Arg-SWNT–Plasmid Complexes

The expression of GFP in transfected root cells was assessed by western blotting. As it was expected (on the basis of fluorescence microscopy observations), western blotting
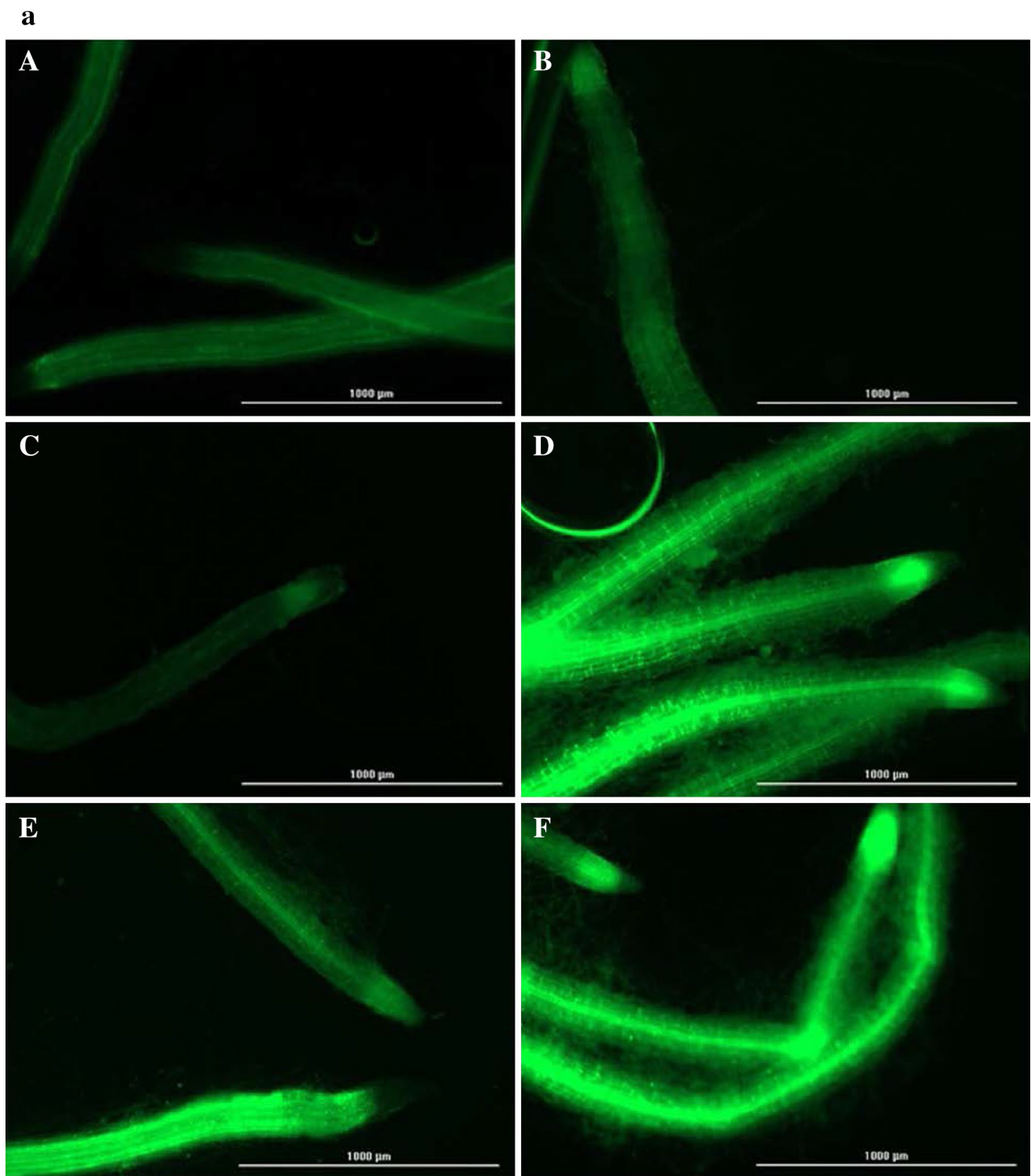
a

Fig. 10 **a** Fluorescent microscopy images of the roots: untreated (**A**), plasmid treated (**B**), peptide treated (**C**), peptide–plasmid complex treated at N/P: 6, 12, 18 (**D**, **E**, and **F**), respectively. The magnifica- tion of the images is ×40. **b** Fluorescent microscopy images of the roots: peptide–plasmid complex treated at N/P: 6, 8, 12, and 18 (**G**, **H**, **I**, and **J**), respectively. The magnification of the images is ×200

analysis of the roots transfected by Arg-SWNT–plasmid complexes illustrated the GFP band (Fig. 12). Surpris- ingly, the existence of GFP protein in root cells transfected

by peptide–plasmid complexes was not verified by west- ern blotting. This finding is in parallel with the RT-PCR results (data not shown) and in contrast with the fluorescent
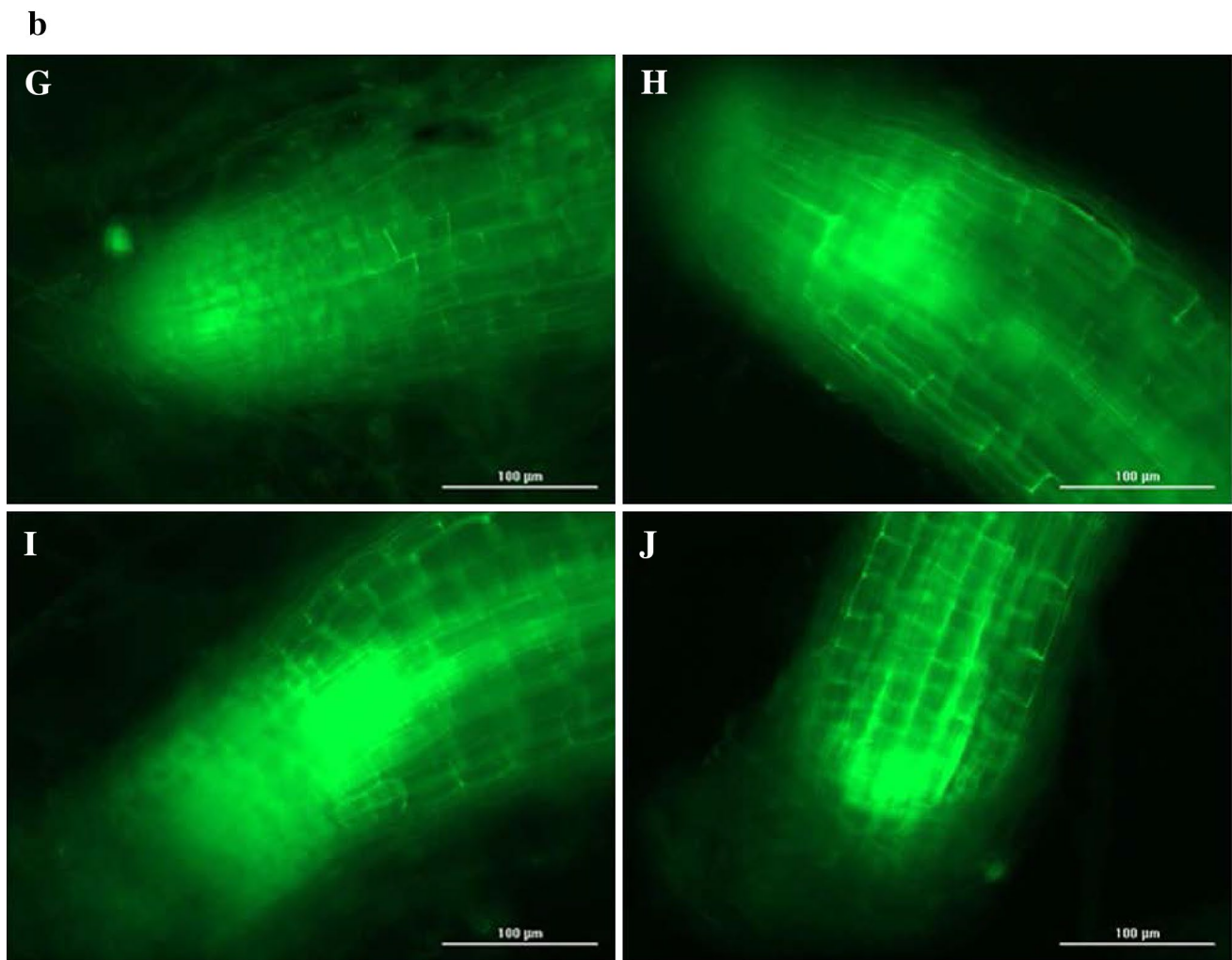
b

Fig. 10 (continued)

microscopy observations. It can be suggested that the presence of shiny spots in the peptide–plasmid complex transfected roots can be due to the accumulation of secondary metabolites such as flavonoids in the root cells. The treatments of plant cells by peptide–plasmid nanoparticles may act as elicitors that provoke plant cells defense system which in turn leads to the production of fluorescent phenolic compounds and antioxidants, such as flavonoids [47–50]. It should be noted that in almost all the previous reports of using CPPs for gene delivery to intact plant cells, the efficiency of transfection was assessed only by fluorescence imaging.

The small diameter of cylindrical SWNTs which is less than the plant cell wall pores (3–5 nm) [51] could account for higher transfection efficiency of carbon nanotubes compared to spherical peptide–plasmid complexes that are more or less larger than normal cell wall pores. In addition, CNT can enter the cells by a needle-like mechanism, with no need for endosomes formation, which increases the chance

of internalization of SWNTs compared to the peptide carriers which encounters endosome as an additional barrier.

Increasing Root Cell Wall Porosity

With the aim of enhancement of peptide–plasmid complex transfection efficiency, we tried to increase root cell wall porosity. Plant cell wall is composed of cellulose and hemicellulose scaffold in pectin matrix. Since cell wall pores are embedded in pectin matrix, treating roots with pectinase may widen the pores [52]. The fluorescence microscopy images illustrated significant differences between pectinase-treated seedlings (as control sample) and pectinase-treated transfected seedlings with peptide–plasmid complexes (Fig. 13), but the lack of GFP band in western blotting analysis was in agreement with the previous results, suggesting the failure of peptide–plasmid nanocarrier to deliver the plasmid inside the plant cell. Treatment of the roots with a mixture of pectinase and cellulase (for a
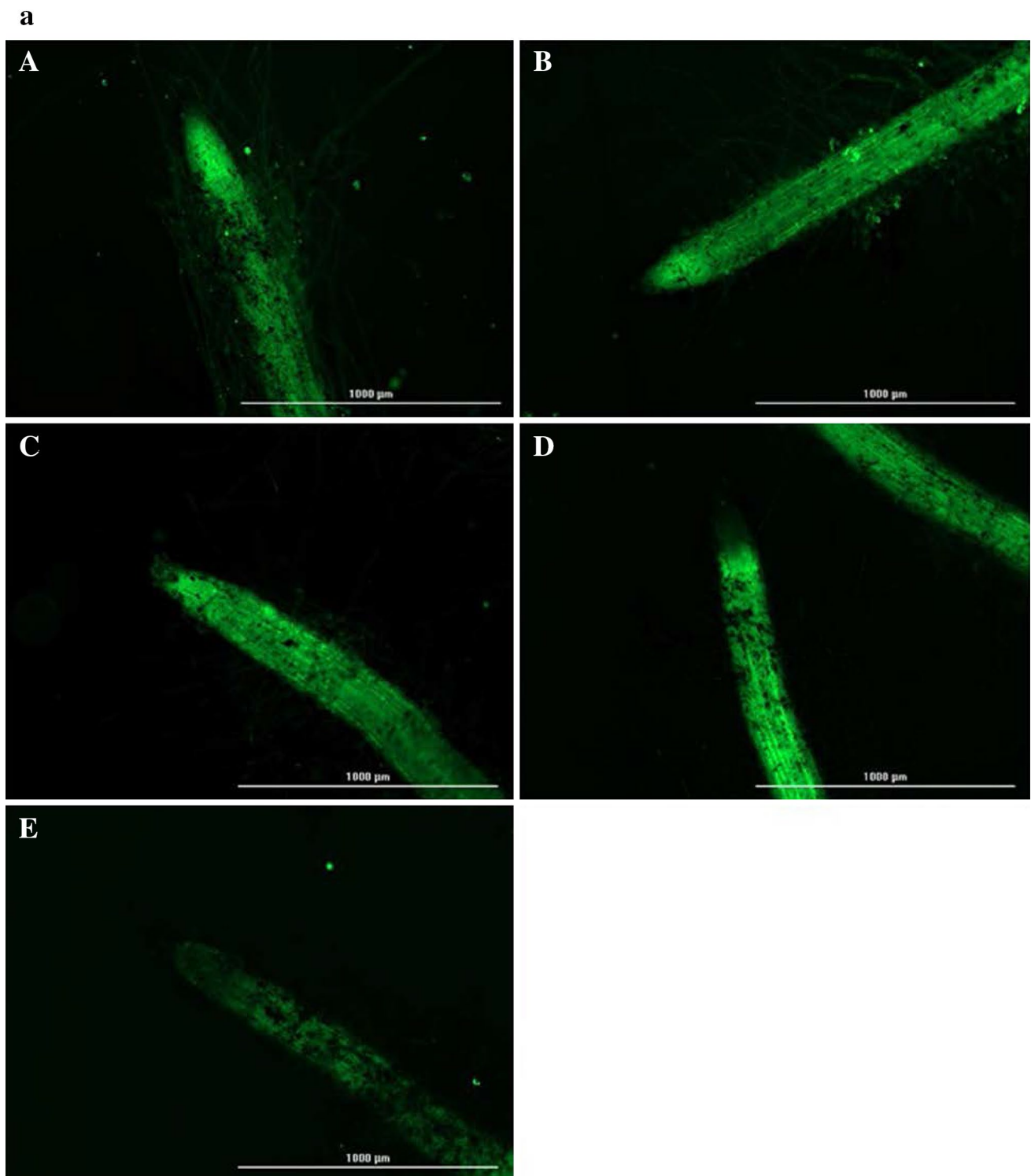
Fig. 11 **a** Fluorescent microscopy images of treated roots with Arg-SWNT–plasmid complexes [10 µg of plasmid in complex with 10, 20, 40, and 80 µg of carbon nanotube (**A**, **B**, **C**, and **D**), respectively], treated root with Arg-SWNT (**E**). The magnification of the images is ×40. **b** Fluorescent microscopy images of treated roots with Arg-SWNT–plasmid complexes [10 µg plasmids in complex with 10, 20, 40, and 80 µg of carbon nanotube (**F**, **G**, **H**, and **I**), respectively], treated root with Arg-SWNT (**J**). The magnification of the images is ×200. **c** Fluorescent microscopy images of treated roots with Arg-SWNT–plasmid complexes: 10 µg of plasmids in complex with 250 µg of carbon nanotube (**A**) 10 µg of plasmid in complex with 400 µg of carbon nanotube (**B**). The magnification of the images is ×200
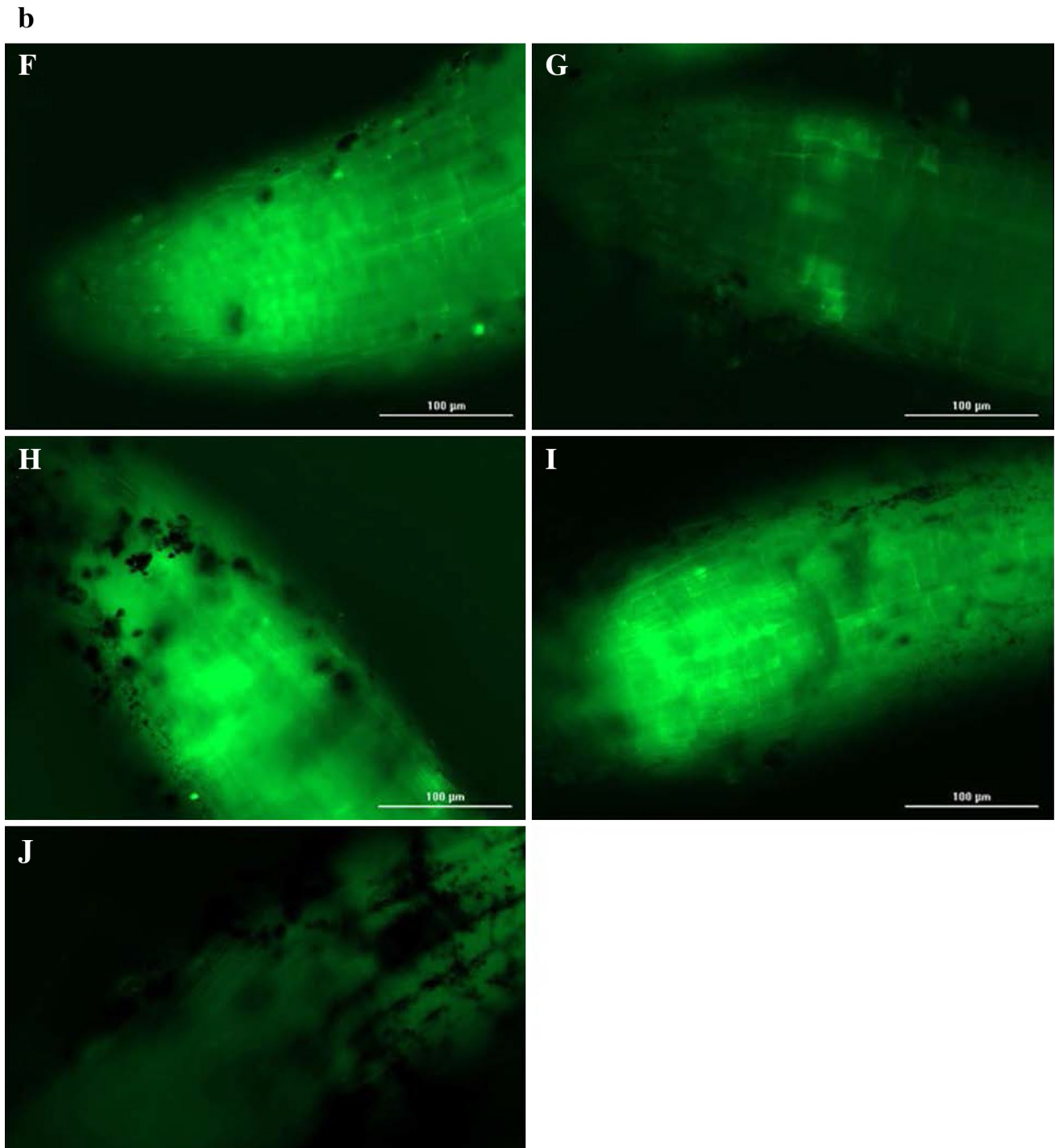
b

Fig. 11 (continued)

short time) did not make significant changes in the results (data not shown).

Another strategy was used to loosen the cell wall by benefiting the expansins proteins. Expansins, which are secreted by plant cells during growth, unlock the network of wall polysaccharides, permitting turgor-driven cell enlargement. They exert a striking effect on elongation and loosening of the cell wall in acidic conditions [53, 54]. Herein, the intact roots transfection by the use of peptide–plasmid structure was done at pH 4 but the expression
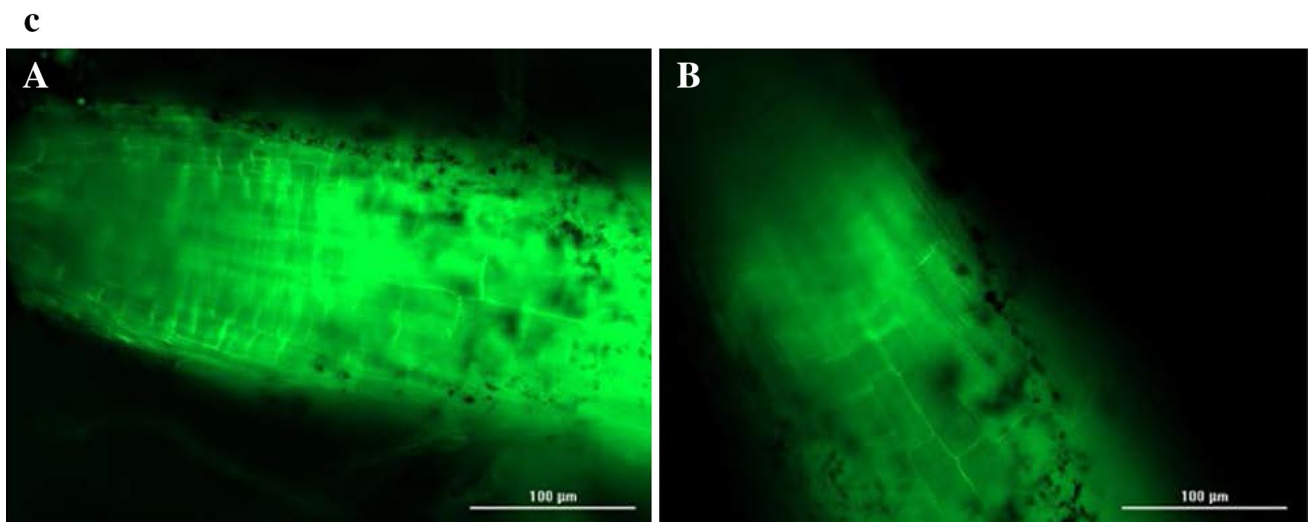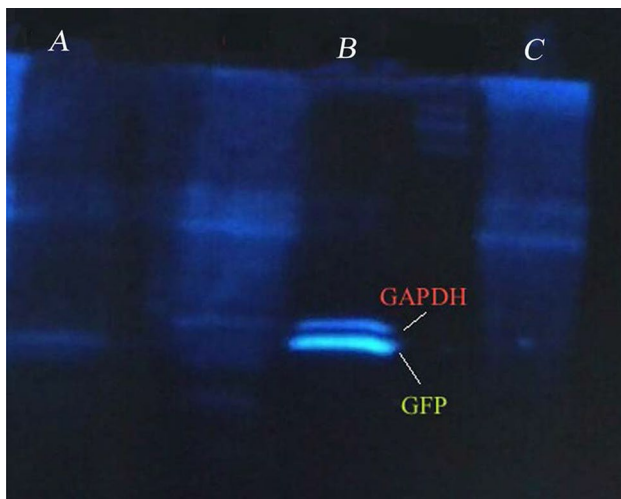
c

Fig. 11 (continued)

Fig. 12 Western blotting of transfected roots by Arg-SWNT–plasmid complexes. (*A*) Transfected roots by Arg-SWNT. (*B*) GFP expressing HEC cell line. (*C*) Transfected roots by peptide

of GFP was not detected by western blotting (data not shown).

As the approaches for prevailing over the cell wall as the first obstacle in gene delivery did not lead to valid evidence of GFP gene expression, we tried to conquer endosome as the next barrier. In order to enhance the endosomal escape of the peptide–plasmid complex,

chloroquine which is a well-known lysosomotropic agent was used. Chloroquine is currently used by many researchers to enhance the delivery of cargos attached to some of the CPPs that are known to be internalized through the endocytic pathway. Despite the impact of chloroquine in accelerating transfection efficiency in the mammalian cell [21, 55], its use at the final concentration of 100 µm on pectinase-treated roots (Fig. 13) did not make any changes in western blotting results.

Conclusion

Overall, in this research engineered chimeric peptides and Arg-SWNTs were tested as easy, fast, and safe gene carriers for tobacco intact root cells transfection. Arg-SWNT could successfully transfer GFP expressing plasmids to root cells, confirmed by fluorescence microscopy images and western blotting analyses. In the case of the engineered chimeric peptides despite the observation of differences between transfected and untransfected roots in fluorescence microscopy images, GFP expression was not confirmed by RT-PCR and western blotting. In order to increase transfection rate by the peptides, different strategies were taken into account to overcome the plant cell barriers although no improvement was observed in the western blotting results.
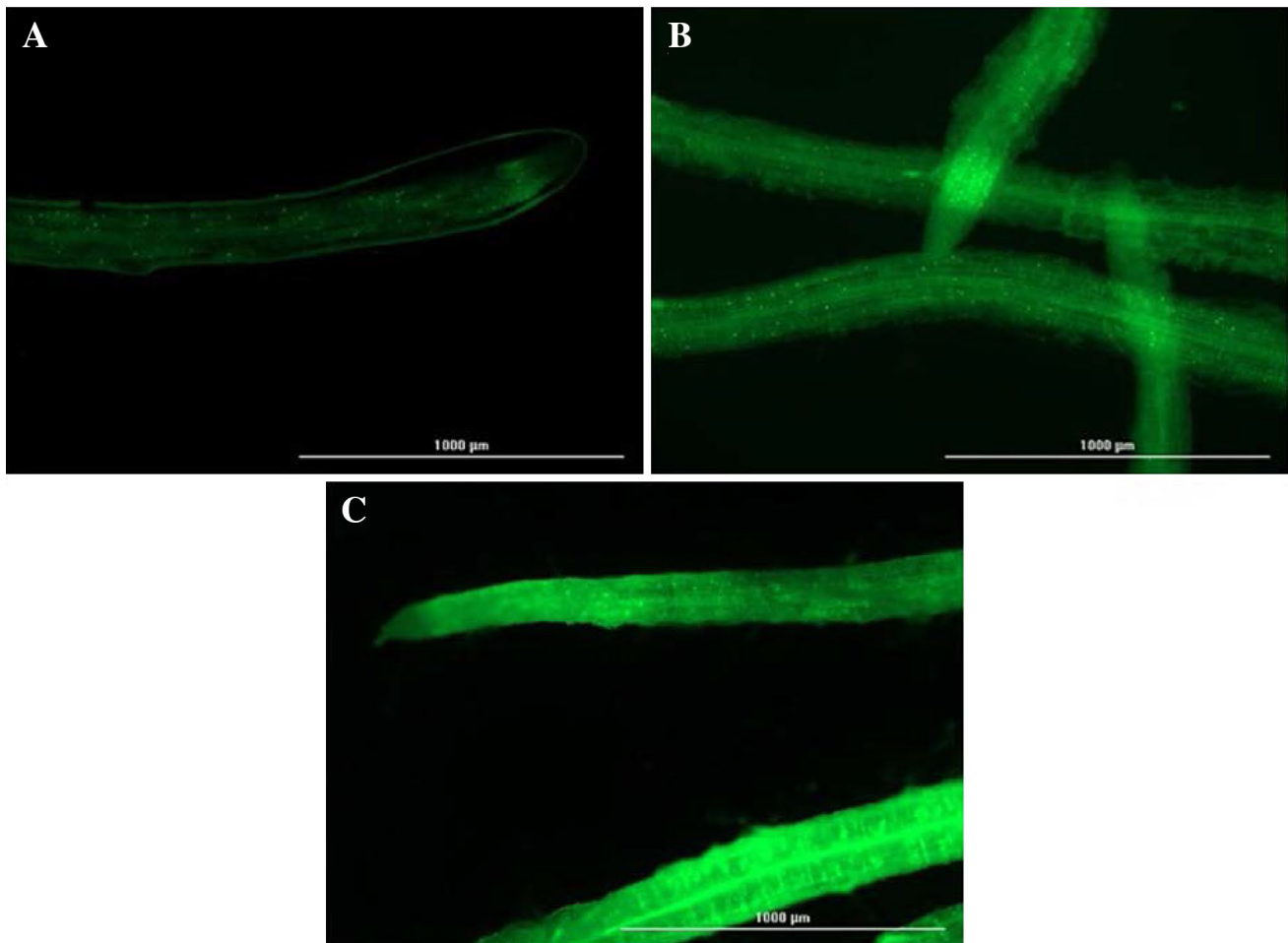
Fig. 13 Fluorescent microscopy images of treated root with pectinase (**A**), treated roots with pectinase, peptide–plasmid complexes and Chloroquine (**B**) and treated roots with pectinase and peptide–plasmid complexes (**C**). The magnification of the images is ×40

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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