Cationic oligopeptide-mediated delivery of dsRNA for post-transcriptional gene silencing in plant cells ☆

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Abstract We have used cationic oligopeptide polyarginine-12mer (POA) to deliver double-stranded RNA (dsRNA), prepared in vitro, to tobacco (*Nicotiana tabacum*) suspension cells. POA interacts electrostatically with dsRNA to form a complex. When dsRNA for the GUS or NPTII gene was delivered into cells carrying the same genes, the corresponding mRNA was degraded. Using RNase protection assay we were able to detect 21-bp small interfering RNA in dsRNA/POA-treated cells. These results demonstrate that POA can be used to deliver dsRNA to induce post-transcriptional gene silencing in plant cells.

Keywords: Polyarginine; Double-stranded RNA; Post-transcriptional gene silencing; RNase protection assay

1. Introduction

Post-transcriptional gene silencing (PTGS) in plants and RNA interference in animals and protozoa, collectively known as RNA silencing, share many components needed to degrade the mRNA homologous to the double-stranded RNA (dsRNA) applied. RNA silencing is thought to be involved in certain developmental or physiological processes in addition to its role in cellular resistance to viral RNA. It has been shown to be effective in a number of organisms including *Drosophila* [1,2], nematodes [3,4], trypanosomes [5], mammals [6] and plants [7,8]. dsRNA, either formed intracellularly or delivered exogenously, plays a central role in triggering degradation of the target mRNA. The dsRNA is cleaved into 21–23 nt small interfering RNA (siRNA) which binds to the RNA-induced silencing complex, also called as RISC, that recognizes the mRNA to be degraded [9–12]. siRNA is amplified by RNA-dependent RNA polymerase (RdRp) to maintain an excess molar ratio over the target mRNA [13]. In order to investigate certain aspects of siRNA amplification in plants, we have sought a procedure by which dsRNA, prepared in vitro, can be effectively delivered into plant cells.

In animal systems, dsRNA is introduced either by injection [3] or by feeding cells in dsRNA-containing medium [14,15]. PTGS has been studied mostly by transforming plants with dsRNA-forming vectors for the selected gene by Agrobacterium [7,8,16,17], by bombardment [18], by infiltration [19], or by infecting plants with viral vectors that produce dsRNA [20]. In addition, Agrobacterium-mediated transient expression was successfully adopted to study PTGS in the intact tissues without generating transgenic plants [21]. Most of these methods require vector construction and plant transformation. In this study, we have developed a cationic oligopeptide to efficiently deliver dsRNA into plant cells.

POA is a modified form of the protein transduction domain (PTD) of the various proteins, including the human immunodeficiency virus-tat transcription factor [22]. PTD in general contains many cationic amino acids, such as arginine or lysine, which are known to play an important role in transverse cell membrane [23]. A β-galactosidase polypeptide, translationally fused to a PTD sequence, was successfully delivered to the most tissues after intraperitoneal injection of mice [24]. It was recently found that due to its cationic nature, PTD can also interact electrostatically with DNA, and that the resulting complex was shown to be delivered effectively into mammalian cells [25,26]. In view of the fact that both dsRNA and DNA are negatively charged, we chose a synthetic cationic homoarginine oligopeptide 12-mer, POA, to deliver dsRNA into tobacco cells to induce PTGS.

2. Materials and methods

2.1. Plant material

Tobacco suspension cells (*Nicotiana tabacum* cv. Petit Havana) were derived from the shoots of transgenic tobacco plants that had been transformed by Agrobacterium with the plasmid pBI121 carrying the GUS and NPTII genes, which were placed downstream of the promoters of 35S and nopaline synthase, respectively. The culture was maintained in the Murashige and Skoog (MS) medium containing 3% sucrose, 2 mg ml^{-1} 2,4-D, 1 mg ml^{-1} kinetin and 50 μg ml^{-1} kanamycin. The culture was routinely transferred to 40 ml MS medium every week and was grown in the dark with gentle shaking (100 rotations per minute (rpm)) at 25 °C.

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Abbreviations: POA, polyarginine oligopeptide; PTGS, post-transcriptional gene silencing; RdRp, RNA-dependent RNA polymerase; FITC, fluorescein isothiocyanate; siRNA, small interfering RNA; EtBr, ethidium bromide; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; rpm, rotation per minute

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2.2. Preparation of dsRNA

To prepare dsRNA, DNA templates were amplified by PCR, introducing T7 RNA polymerase binding sequences at the 5′ end of the both strands. The 0.9- and 0.4-kb internal regions of GUS and NPTII coding sequences, respectively, were amplified. The products were transcribed in both the directions by T7 RNA polymerase using the Megascript T7 Transcription Kit (Ambion, USA) to generate both sense and antisense RNAs. After the DNA templates were removed by DNase, RNA was purified by phenol–chloroform extraction and was precipitated by ethanol. The resulting pellet was suspended in DEPC-treated water and annealed into dsRNA at 65 °C. The dsRNA was quantified using GeneQuant (Pharmacia Biotech, USA) and stored at −20 °C.

2.3. Preparation and delivery of fluorescein isothiocyanate-dsRNA

To prepare fluorescein isothiocyanate (FITC)-dsRNA (GUS), dsRNA was prepared as described above, except that aminoallyl UTP (Sigma, USA) was included instead of UTP. FITC was then conjugated to the dsRNA by mixing and stirring the dsRNA with 150 μg FITC/mg dsRNA in bicarbonate buffer (pH 9.5) at 4 °C overnight. The FITC-dsRNA was precipitated with sodium acetate/ethanol and purified via a Sephadex G-50 column. The FITC-dsRNA thus prepared was complexed with POA before being delivered to cells.

2.4. Formation of dsRNA/POA complex

POA was commercially synthesized (Peptron Inc., Korea). POA was dissolved in phosphate-buffered saline (PBS, pH 7.4) at a concentration of 200 μM and aliquots were frozen at −70 °C until use. To determine the optimal binding ratio of dsRNA to POA, 100 ng of dsRNA (GUS) was mixed with the various concentrations of POA (5, 10, 15, 20, 25, 37.5 and 50 ng) in PBS buffer to a final volume of 10 μl and incubated on ice for 30 min and electrophoresed in 1% agarose gel. The point at which the dsRNA disappeared was taken as the optimum amount of POA and was scaled up for further studies. The optimum concentration of POA for dsRNA (NPTII) was determined in the same way.

2.5. Delivery of dsRNA/POA complex to suspension cells

Various concentrations of dsRNA of GUS or NPTII (1, 5, 10 and 20 μg) were mixed with POA and the dsRNA/POA complex was delivered to suspension cells. MS medium was removed from 5 ml of 3-day-old suspension cell (approximately 1 g fresh cell) and cells were rinsed with PBS. Cells were mixed with dsRNA/POA complex in a volume of 500 μl and these mixtures were shaken (100 rpm) for 1 h at 25 °C. Then, 3 ml of fresh MS medium (free of dsRNA) containing 50 $\mu g\,ml^{-1}$ kanamycin was added and incubated for the designated periods. As the controls, cells treated with PBS only, POA only (10 μg), and dsRNA only (20 μg) were included. All the PTGS experiments were done at least three times to confirm the results.

The viability of the treated cells was measured by 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) calorimetric assay after 3-week of treatment. To the equal amount of cells (ca. 1 g) 20 μl of MTT (5 $mg\,ml^{-1}$) in PBS buffer was added and incubated at 25 °C for 5 h. Later, cells were sonicated and 50 μl of lysis solution (20% SDS, 50% dimethyl formamide) was added and incubated for 2 h in the dark at room temperature. After the incubation, the cell suspension was centrifuged and the supernatant measured at 570 nm.

2.6. RT-PCR

Total RNA was prepared from the samples using TRI reagent (Molecular Probes Diagnostics, USA) following the manufacturer's instructions. After the medium was removed, cells were homogenized with 1 ml of TRI reagent. To the homogenized cell suspension, bromochloro propane was added. Following incubation for 15 min, the mixture was centrifuged at $12\,000\times g$ for 15 min. The RNA from the aqueous phase was precipitated with isopropanol. About 5 μg of total RNA was used to prepare cDNA using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Oligo$(dT)_{18}$ primers were used for cDNA synthesis. 5 μl of cDNA was amplified for 30 cycles at 58 °C as the annealing temperature. The primers used were the same as those used to prepare the templates for the dsRNA. 5 μl of each amplified fragment was analyzed on a 1% agarose gel electrophoresis.

2.7. Detection of siRNA by RNase protection assay

Total RNA was prepared from cells with TRI reagent as described above with slight modifications. Total RNA was precipitated with yeast tRNA and ethanol for 30 min at −70 °C, dissolved in DEPC-treated water. The small size RNA was enriched [27] and it was used for the RNase protection assay [28]. In brief, the enriched small RNA was hybridized to [α-^{32}P]UTP-labeled sense-strand RNA (ssRNA) (the 900-bp RNA which corresponds to the internal region of GUS) overnight at 35 °C. siRNA in the enriched fraction binds to the ssRNA during this period. The unhybridized single-stranded RNA was digested with 40 $\mu g\,ml^{-1}$ RNase A and 500 U RNase T1 (Ambion, USA) in RNA digestion buffer (300 mM NaCl, 10 mM Tris–Cl at pH 7.4 and 5 mM EDTA at pH 7.5) at 37 °C for 1 h. The reaction was stopped by adding 20 μl of 10% SDS and 10 μl of proteinase K, and incubated at 30 °C for 30 min. The mixture was purified with phenol–chloroform and precipitated with yeast tRNA at −70 °C for 30 min, followed by fractionation on a 15% denaturing PAGE gel and exposure to X-ray film for 24 h. In vitro-prepared small 21-nucleotide antisense RNA was used for the marker.

3. Results

3.1. Localization of FITC-POA and FITC-dsRNA into cells

Prior to delivery of dsRNA into tobacco cells, localization of both FITC-labeled dsRNA (FITC-dsRNA) and FITC-POA was examined (Fig. 1). It was observed that FITC-POA was located near the nuclear membrane and within the nucleus (A). POA and FITC-dsRNA were allowed to form a complex before being delivered into cells. The FITC fluorescence of dsRNA was shown to gradually disperse from the nuclear membrane (B) to the cytoplasm (C). Since PTGS is likely to take place in the cytoplasm where mRNA is localized, overnight incubation of dsRNA was thought to be suitable to initiate PTGS. It was also noted that dsRNA was not transported to the nucleus, as FITC fluorescence was not observed there (C).

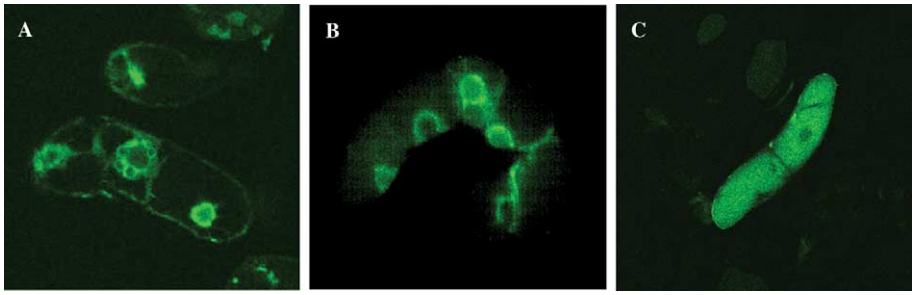

Fig. 1. Localization of FITC-POA and FITC-dsRNA. 3-day-old tobacco suspension cells were incubated with FITC-POA (A), or with POA complexed with FITC-dsRNA (GUS) for 1 h (B) or 16 h (C), and localization of FITC fluorescence was observed with a fluorescence microscope.

3.2. dsRNA/POA complex formation

To establish the optimum amount of the POA needed for interacting dsRNA (GUS), various concentrations of POA were mixed with dsRNA (GUS) to form a complex, as described in Section 2, and these mixtures were subjected to 1% agarose gel electrophoresis to examine the mobility retardation. As the amount of POA increased, the mobility of the dsRNA decreased (Fig. 2), indicating that dsRNA was complexed with POA. It was also noted that ethidium bromide (EtBr) staining of the dsRNA also weakened as the POA concentration increased. When 100 ng of dsRNA and 50 ng of POA were mixed, there was no EtBr staining, strongly suggesting that dsRNA was protected by POA (lane 9). This suggestion was further supported by the observation of reappearance of staining after addition of β-mercaptoethanol (lane 10), which disrupted the interaction between dsRNA and POA to allow dsRNA to move equivalent to free dsRNA (lane 1). This structure was proposed in the complexes of DNA and cationic oligopeptides in general [26].

Scaling up the ratio used in lane 9, 20 μg dsRNA (GUS) and 10 μg POA were mixed and were allowed to interact for 30 min in 500 μl PBS in most of the experiments unless otherwise specified. It was assumed that all the dsRNA was bound to POA in the complex-forming condition mentioned above, since no free-dsRNA was detected after forming the complex (data not shown). Several repeated experiments showed that 20 μg dsRNA, complexed with POA, was sufficient to induce PTGS in approximately 1 g of tobacco suspension cells in 3 ml total volume (34 pM of dsRNA).

3.3. Induction of PTGS

To investigate if dsRNA/POA (GUS or NPTII) induces PTGS, we examined the degradation of the corresponding mRNAs in cells treated with a given dsRNA (Fig. 3). To silence GUS, various amounts of dsRNA (GUS), complexed with POA, were incubated with cells for 24 h. Northern blot analysis showed that 20 μg of dsRNA appeared to be sufficient to degrade GUS mRNA completely (Fig. 3A). RT-PCR results gave the similar results (Fig. 3B). NPTII mRNA appeared to be stable during dsRNA (GUS) treatment (Fig. 3B), indicating that dsRNA degradation was specific. In an equivalent way, when dsRNA(NPTII) was delivered, NPTII mRNA was degraded and GUS mRNA unaffected (Fig. 3C). 10 μg of dsRNA (NPTII) appeared to degrade mRNA (NPTII) completely within 72 h (Fig. 3C). It was also shown that the POA-, dsRNA- or PBS-alone controls did not affect both GUS and NPT II mRNAs.

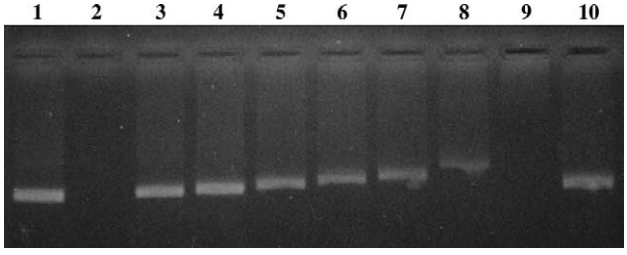

Fig. 2. Formation of POA/dsRNA complex. dsRNA (GUS) was complexed with POA as described in Section 2 and was analyzed on a 1% agarose gel to determine the optimum POA concentration for the probable penetration-effective complex: lane 1, 100 ng dsRNA (GUS); lane 2, POA alone (50 ng); lanes 3–9: 5, 10, 15, 20, 25, 37.5 and 50 ng of POA mixed with dsRNA; lane 10, same as lane 9 except including β-mercaptoethanol in the loading dye.

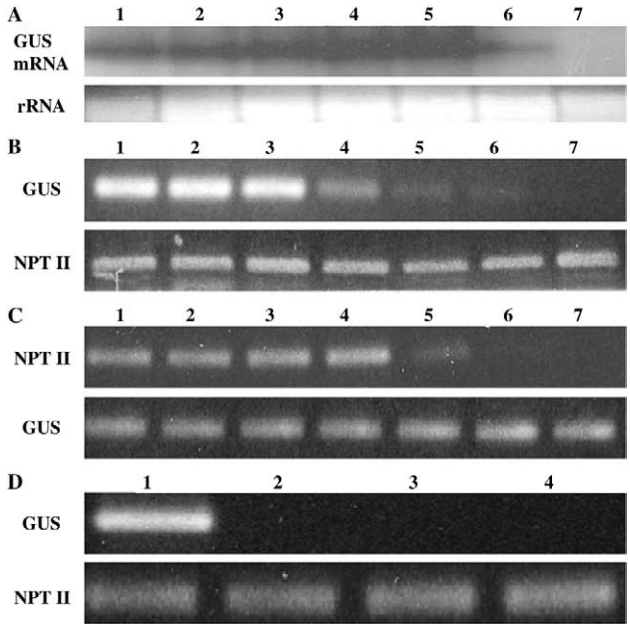

Fig. 3. PTGS of GUS and NPTII mRNA in POA/dsRNA-treated cells. For Northern blot analysis, total RNA (20 μg) prepared from cells treated with POA/dsRNA (GUS) was probed with ^{32}P-labeled 0.1-kb EcoRV fragment within GUS coding region (A). RT-PCR analysis of GUS mRNA (B). Different amounts of dsRNA (GUS) were treated for 24 h to examine degradation of GUS mRNA: lane 1, no dsRNA treatment; lane 2, POA alone; lane 3, dsRNA alone; lanes 4–7, 1, 5, 10 and 20 μg dsRNA complexed with POA, respectively. The EtBr stain of rRNA is shown. RT-PCR analysis of NPTII mRNA (C). Different amounts of dsRNA (NPTII) were treated for 72 h to examine degradation of NPTII mRNA. Lanes equivalent as in (B), dsRNA (NPTII) instead of dsRNA (GUS). Longevity of PTGS (D). To examine duration of PTGS, cells were exposed to 20 μg dsRNA (GUS) for 1 h, followed by further incubation in the absence of dsRNA for 1 (lane 2), 2 (lane 3) and 3 (lane 4) weeks (lane 1, 3-week incubation of the POA-only control).

Many PTGS studies have used transformed plants in which dsRNA was stably formed by chromosome-integrated inverted repeat DNA construction. Since dsRNA was added exogenously in this study, it was assumed that PTGS would be transiently expressed according to the presence of dsRNA in the cells, expecting that silenced target mRNA appeared again as dsRNA disappeared. After dsRNA (GUS) treatment for 1 h, there was no indication of the reappearance of GUS mRNA, suggesting that the PTGS lasted for at least 3 weeks. Gentle shaking and medium changes every 5 days prevented cells from deterioration, as evidenced by MTT cell viability assay (data not shown). It was found that there was no indication of re-appearance of GUS mRNA (Fig. 3D), suggesting that PTGS lasted for at least 3 weeks. NPTII mRNA was unaffected during this period (Fig. 3D), further suggesting that cells had maintained viability during this time. Both GUS and NPT II mRNAs appeared to be persistent in the POA-treated control cells that were grown also for three weeks (Fig. 3D).

3.4. Formation of siRNA

We further substantiated the occurrence of PTGS by showing the presence of siRNA using RNase protection assay. siRNA, corresponding to the 21-nt marker, was detected only

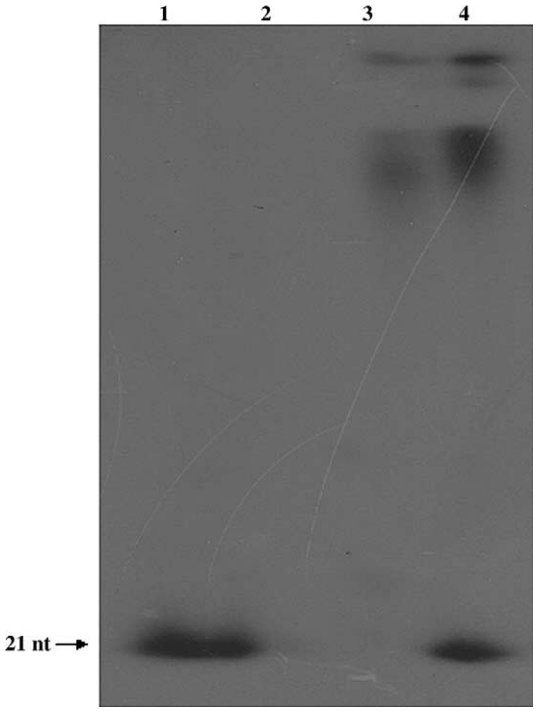

Fig. 4. siRNA detection by RNase protection assay. Cell was treated with dsRNA (GUS) and total RNA was prepared. The enriched small RNA fraction was hybridized to the ^{32}P-labeled sense strand of GUS. Unhybridized single strand portion of probe was removed by ssRNA-specific RNaseA and remaining siRNA was detected by autoradiogram: lane 1, 21-nt marker; lane 2, PBS-only; lane 3, cells treated with dsRNA alone and lane 4, cells treated with dsRNA/POA.

in cells treated with POA/dsRNA (GUS) and not in the dsRNA-alone control (Fig. 4). We have thus demonstrated that POA delivered dsRNA effectively and conveniently to induce PTGS in tobacco suspension cells.

4. Discussion

In this study, we observed efficient silencing of GUS and NPTII genes by the respective dsRNA complexed with POA. POA efficiently delivered the dsRNA into tobacco cells to induce PTGS. It is likely that delivery of dsRNA can be improved by altering lengths and/or amino acid make-up of the peptides. It was suggested that the longer polypeptides have so many positive charges interacting with the mRNA that the complexes are too tight to dissociate inside the cell [29]. It was also suggested that small peptides with only 12–16 positive charges released DNA easily in the cells [29]. These notions may explain that POA, composed of 12 arginines, is an efficient carrier of dsRNA.

The prolonged PTGS in dsRNA/POA-treated cells suggests that silencing was spread from silenced to non-silenced cells. It is also likely that amplification of siRNA by RdRp by repeated rounds of dsRNA synthesis [30] may be the cause of the observed prolonged silencing. The probable correlation between RdRp activity and longevity of PTGS is presently being investigated.

Although PTD has been widely used for gene delivery mostly in animal cells, this technology has not been reported in plant studies. Our results showed that POA did not deteriorate plant cells at least for 3 weeks in the growth condition used in this study. It is suggested that one of the prevalent applications of PTGS is to study the function of gene by degrading the corresponding mRNA. If one can readily induce PTGS in cells, PTGS can be a strong alternative to the widely used methods such as T-DNA insertional mutagenesis. In view of convenience of preparation and delivery of dsRNA, POA-mediated PTGS technology may be applied to the studies of functional genomics or some other biotechnological researches.

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