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Enhanced Delivery of siRNA Complexes by Sonoporation in Transgenic Rice Cell Suspension Cultures

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Small interfering synthetic double-stranded RNA (siRNA) was applied to suppress the expression of the human cytotoxic-T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) gene transformed in transgenic rice cell cultures. The sequence of the 21-nucleotide siRNA was deliberately designed and synthesized with overhangs to inactivate the expression of hCTLA4Ig. The chemically synthesized siRNA duplex was combined with polyethyleneimine (PEI) at a mass ratio of 1:10 (0.33 μg siRNA:3.3 μg PEI) to produce complexes. The siRNA complexes (siRNA+PEI) were labeled with Cy3 in order to subsequently confirm the delivery by fluorescent microscopy. In addition, the cells were treated with sonoporation at 40 kHz and 419 W for 90 s to improve the delivery. The siRNA complexes alone inhibited the expression of hCTLA4Ig to 45% compared with control. The siRNA complexes delivered with sonoporation downregulated the production of hCTLA4Ig to 73%. Therefore, we concluded that the delivery of siRNA complexes into plant cells could be enhanced successfully by sonoporation.

Keywords: Polyethyleneimine, transgenic plant cell cultures, siRNA, sonoporation, hCTLA4Ig

Transgenic plant cell cultures are considered as an attractive model system for producing plant-made pharmaceuticals (PMPs) [8, 14]. PMPs include various human and animal proteins of pharmaceutical value. Transgenic plant cell culture systems for the production of PMPs have several advantages over the other kinds of hosts. They provide a stability of supply, low production costs, safety due to the human pathogen-free process, capability for post-translational modifications, ease of scale-up, and simple media compositions [21]. Recently, transgenic rice cells produced recombinant human proteins, using the RAmy3D promoter, such as granulocyte-colony stimulating factor [9], granulocyte–macrophage colony stimulating factor [22], serum albumin [10], α_1-antitrypsin [18], and interferon-gamma [1]. In the RAmy3D promoter system, expression is strongly regulated by sugar starvation.

Small interfering RNA (siRNA) is 19 to 23 nucleotides of double-stranded RNA that can induce RNA silencing. RNA silencing is also known as post-transcriptional gene silencing, (PTGS) in plants, RNA interference (RNAi) in mammals and quelling in fungi [19, 20]. Strategies for RNAi are classified according to chemical synthesis, *in vitro* enzymatic synthesis, DNA plasmid vector, and virus-mediated vector [3]. Chemically synthesized siRNA duplexes are highly pure, easy to prepare, and can be rapidly delivered directly into cells, compared with the other methods [27]. According to the level of homology, siRNA can either disassemble a specific mRNA or inhibit translation of a target mRNA [20, 26]. Since siRNA can inhibit the expression of any gene, it is possible to identify the function of novel genes with this technique [32]. siRNA-mediated gene silencing was established to silence GFP expression in cultured *gfp* transgenic cells of rice, cotton, fir, and pine [27].

Sonoporation is an ultrasound (>20 kHz) treatment that can temporarily enhance the permeability of cell membrane, thereby facilitating the uptake of nucleic acids [15]. Permeability enhancement by sonoporation can be transiently generated by acoustic cavitation as a nonthermal effect [11]. It was reported that plasmid DNA could be delivered into sugar beet protoplasts by 20 kHz ultrasound at 0.5–1.5 W/cm^2 of acoustic power. Furthermore, sonoporation increased the delivery of exogenous DNA into insect organs *in vivo* [12].

In this study, recombinant human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) was targeted for disruption because it is unique in transgenic rice cells. A transgenic rice cell line expressing hCTLA4Ig [2, 13] was used, and the degree of inhibition in hCTLA4Ig expression could also be easily estimated by ELISA. Owing to the

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uniqueness of the target mRNA, it was possible to avoid off-target silencing through the specific design of the siRNA sequence. On the other hand, because synthetic siRNA is easily degraded by the nucleases in the culture medium and is poorly transferred into cells, there are certain obstacles that must be overcome regarding the instability and low efficiency of siRNA delivery. siRNA alone cannot enter plant cells because cell walls are too rigid and thick. Hence, siRNA must be combined with transfection vectors for gene delivery in this system. One of the vectors is polyethyleneimine (PEI), which has been used as a nonviral vector to facilitate the uptake of nucleic acids [16]. Until now, there have been no studies on the application of chemically synthesized siRNA to suppress the expression of a specific transformed gene in transgenic plant cell cultures. We developed a delivery method to inhibit the expression of a target mRNA. siRNA was combined with PEI to form siRNA complexes, and sonoporation was used to improve the delivery of the complexes into the cultured plant cells.

Materials and Methods

Plant Cell Cultures

The transgenic rice (*Oryza sativa* L. cv. Dongjin) cell line expressing hCTLA4Ig was established by Boryung Pharmaceutical Co. Ltd. (Ansan, Korea) as previously reported [13]. Transgenic rice cells were maintained in 500-ml Erlenmyer flasks with 140 ml of fresh medium on a shaking incubator at 120 rpm under dark condition and the culture temperature was 28°C. The cells were maintained in an AA medium containing 2,4-dichlorophenoxyacetic acid (2 mg/l), kinetin (0.2 mg/l), and sucrose (30 g/l). The suspension cells were transferred to fresh medium every 10 days.

Table 1. The designed sequence of siRNA for hCTLA4Ig.

1	ATGCAGGTGC TGAACACCAT GGTGAACAAA CACTTCTTGT CCCTTTCGGT
51	CCTCATCGTC CTCCTTGGCC TCTCCTCCAA CTTGACAGCC GGGGCAATGC
101	ACGTGGCCCA GCCTGCTGTG GTACTGGCCA GCAGCCGAGG CATCGCCAGC
151	TTTGTGTGTG AGTATGCATC TCCAGGCAAA GCCACTGAGG TCCGGGTGAC
201	AGTGCTTCGG CAGGCTGACA GCCAGGTGAC TGAAGTCTGT GCGGCAACCT
251	ACATGATGGG GAATGAGTTG ACCTTCCTAG ATGATTCCAT CTGCACGGGC
301	ACCTCCAGTG GAAATCAAGT GAACCTCACT ATCCAAGGAC TGAGGGCCAT
351	GGACACGGGA CTCTACATCT GCAAGGTGGA GCTCATGTAC CCACCGCCAT
401	ACTACCTGGG CATAGGCAAC GGAACCCAGA TTTATGTAAT TGATCCAGAA
451	CCGTGCCCAG ATTCT

Position in gene sequence (418)	5'-AACGGAACCCAGATTTATGTA-3'
GC content	38.1%
Sense strand siRNA	CGGAACCCAGAUUUAUGUAtt
Antisense strand siRNA	UACAUAAAUCUGGGUUCCGtt

Estimation of Cell Viability

The cell viability was evaluated by the TTC (2,3,5-triphenyltetrazolium chloride) method. For the reaction, 1.6 ml of TTC solution [1% (w/v) TTC in KH_2PO_4 buffer, pH 9.0] was added into 0.1 g of fresh cells. After 24 h at 20°C under dark condition, the supernatants were removed by centrifugation at 13,000 rpm for 15 min. Red formazan was then extracted from the cells by incubation with 1 ml of 95% ethanol at 60°C for 30 min. The formazan-containing supernatants were then separated by centrifugation at 13,000 rpm for 30 min. The absorbance was measured using an Agilent 8453 spectrophotometer (Agilent Technologies Inc., U.S.A.) at 485 nm to analyze the relative cell viability.

siRNA Design and Synthesis

The sequence of siRNA was designed using the siRNA Target Finder. The designed sequence is given in Table 1. The siRNA contained 21 nucleotides with 3'-dTdT overhangs. These nucleotides were located at position 418 in the hCTLA4Ig gene sequence and included 38.1% GC content. A BLAST (basic local alignment search tool) search was also performed with this sequence to avoid off-target silencing in the rice cells. The designed siRNA sequence was synthesized by Ambion, Inc. (U.S.A.).

Sonoporation Treatment

The sonoporation treatment was performed using a Bransonic 5210 (Branson Ultrasonics Corp., U.S.A.) sonicator at 40 kHz and 419 W for 90 s. Ultrasound can cause alternating high and low pressures in solution. During this alternation, millions of microbubbles are formed and grown [11]. This is called cavitation, meaning the formation of cavities.

Fluorescence Microscopic Assay

The delivery of Cy3-labeled siRNA complexes was confirmed by fluorescence microscopy. For the analysis, 0.01 g of the cells was suspended in 0.5 ml of medium. Samples were then settled in ice for 15 min. Three μl of Cy3-labeled siRNA was added to the samples and the reaction was performed with mild vortexing for 2 h. The mixture was centrifuged at 1,600 ×*g* for 5 min at 4°C. After the removal of supernatants, the pelleted cells were washed 3 times with chilled medium to detach any Cy3-labeled siRNA remaining on the cell surface. The cells were then resuspended in medium and observed by fluorescence microscopy.

Measurement of Recombinant hCTLA4Ig

The hCTLA4Ig expression level was measured by enzyme-linked immunosorbent assay (ELISA). Culture broth was centrifuged at 4°C and 12,000 rpm. The supernatant was stored at −70°C before use. For sandwich ELISA, 96-well microplates were coated with goat anti-human IgG Fc (1:1,000; KPL Inc., U.S.A.) and blocked with phosphate-buffered saline containing 2% fetal bovine serum and 0.05% Tween 20. Each well was loaded with sample or protein standards, ranging from 11 to 0.08 ng/ml in a series of eight 2-fold dilutions. Peroxidase-labeled goat anti-human IgG (γ) (1:5,000; KPL Inc., U.S.A.) was applied as the detection antibody, followed by the horseradish-peroxidase (HRP) substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-

sulfonic acid) (ABTS, KPL Inc., U.S.A.). Model 550 microplate reader (Bio-Rad Laboratories Inc., U.S.A.) was used to measure the absorbance at 405 nm.

RESULTS

Formation of siRNA Complexes

To confirm the formation of siRNA complexes by the combination of siRNA with PEI, a gel retardation assay was performed. As shown in Fig. 1, siRNA complexes in lanes 4 and 6 were not stained by ethidium bromide (EtBr). Since PEI could interrupt the EtBr staining for the visualization of siRNA, siRNA complexes were not completely detected by EtBr. Additionally, sonoporation treatment in lanes 5 and 6 did not affect the stability of siRNA complexes.

Effect of siRNA Complexes on Cell Viability

Effects of the addition of siRNA complexes on cell viability were investigated. siRNA complexes were formed by combining siRNA and PEI at a mass ratio of 1:10. Sonoporation at 40 kHz and 419 W for 90 s was immediately performed on day 0. Since the RAmy3D promoter system is inducibly regulated by sugar starvation, cell viability of the control cells without siRNA dropped to 68% by 4 days after induction because of the complete absence of sugar. Dry cell weight was reduced from 7.8 g/l on day 0 at the time of induction to 5.8 g/l on day 4. As shown in Fig. 2, formation of siRNA complexes and application of sonoporation did not influence cell viability during the induction period of the culture.

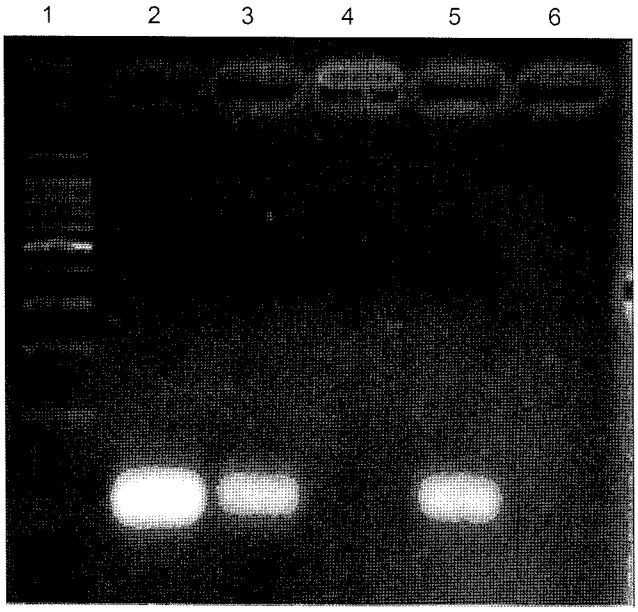

Fig. 1. Visualization of siRNA complexes by gel retardation assay: lane 1, marker; lane 2, siRNA only; lane 3, Cy3-siRNA; lane 4, Cy3-siRNA+PEI; lane 5, Cy3-siRNA+ultrasound; lane 6, Cy3-siRNA+PEI+ultrasound.

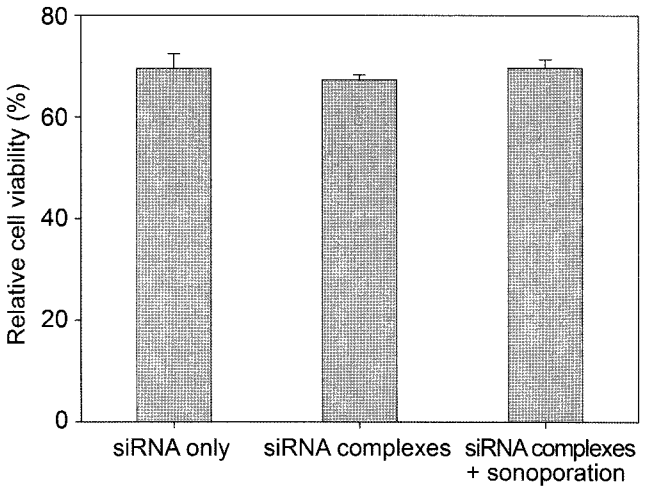

Fig. 2. Effects of the formation of siRNA complexes and sonoporation on cell viability.

Comparison of Delivery Methods

Cy3-labeled siRNA was transferred by different delivery methods into plant cells and the fluorescence microscopic results are shown in Fig. 3. Cy3-siRNA alone (A) and Cy3-siRNA with sonoporation (B) did not enter into plant cells efficiently. However, siRNA complexes (C) combined with PEI were delivered into plant cells more effectively. Moreover, the uptake of siRNA complexes with sonoporation (D) increased the uptake of siRNA complexes significantly.

Effect of PEI on the Stability of Extracellular hCTLA4Ig

To evaluate whether PEI interfered with the quantitative analysis of hCTLA4Ig, the effects of PEI on the stability of hCTLA4Ig were investigated. Since 3.3 μg of PEI was combined with 0.33 μg of siRNA, the concentrations of PEI used in this experiment ranged from 4 to 400 μg/l. Fig. 4 shows the influence of different concentrations of PEI on the stability of hCTLA4Ig. Considering the maximum hCTLA4Ig production (15 mg/l, 6 days after induction), the addition of 4 to 400 μg/l of PEI to the culture medium did not affect the stability and the quantitative analysis of hCTLA4Ig.

Time-Course Profiles of hCTLA4Ig Expression with the Presence of Various Forms of siRNA

The patterns of hCTLA4Ig gene silencing were observed during the induction period. The production of hCTLA4Ig in control cells without siRNA treatment peaked at 13.6 mg/l after 6 days (Fig. 5). The production of hCTLA4Ig increased steadily up to 8.2 mg/l in the cultures treated with siRNA complexes, but rose slightly up to 3.4 mg/l in the cultures treated with siRNA complexes and sonoporation. The siRNA complexes delivered with sonoporation could completely downregulate the expression of hCTLA4Ig until day 2. siRNA alone could not inactivate the expression of

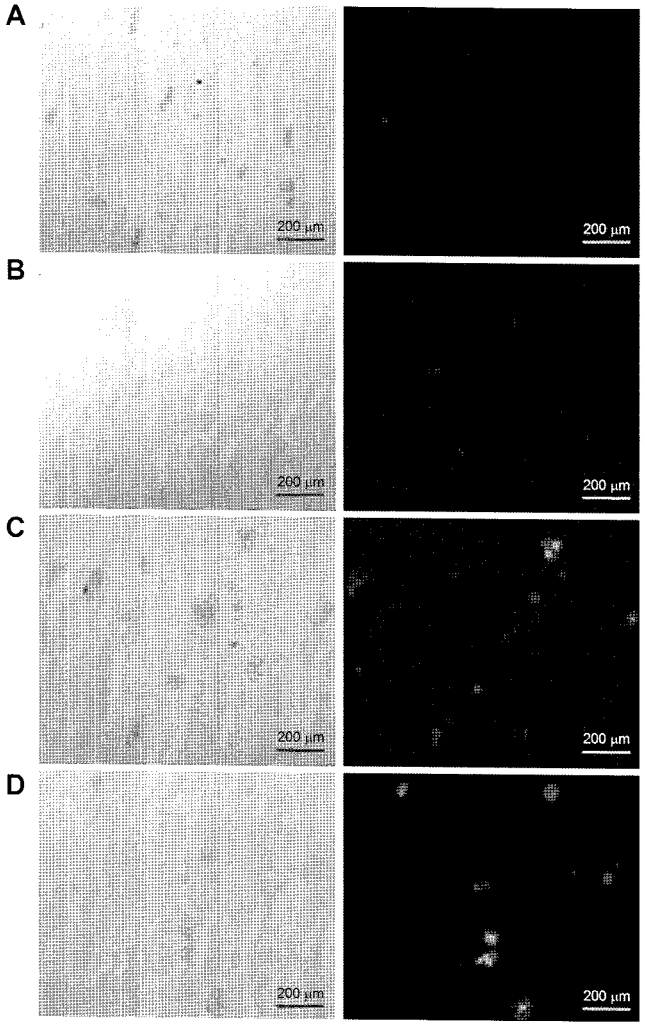

Fig. 3. Comparison of Cy3-labeled siRNA complex delivery methods using fluorescence microscopy: (**A**) Cy3-siRNA only; (**B**) Cy3-siRNA+ultrasound (90 s); (**C**) Cy3-siRNA+PEI (1 h); (**D**) Cy3-siRNA+PEI (1 h)+ultrasound (90 s).

hCTLA4Ig during the induction period, and the level of produced hCTLA4Ig was almost the same as that of the control culture. Consequently, siRNA complexes inhibited

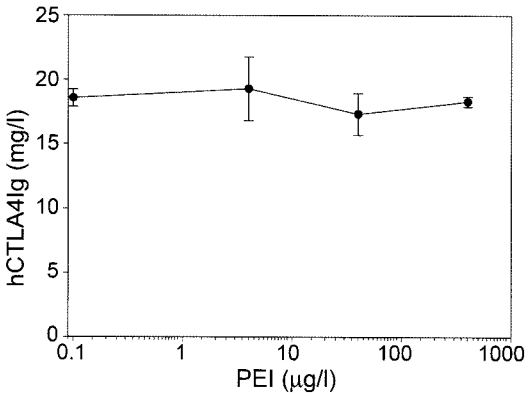

Fig. 4. Influence of PEI concentrations on the stability of extracellular hCTLA4Ig.

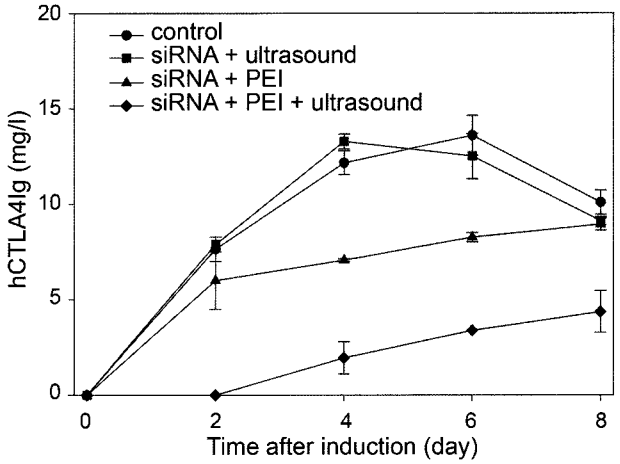

Fig. 5. Time-course changes of hCTLA4Ig expression after siRNA treatment: (●) siRNA only; (■) siRNA+ultrasound; (▲) siRNA+PEI; (◆) siRNA+PEI+ultrasound.

the production of hCTLA4Ig by 45%. Additionally, the siRNA complexes delivered with sonoporation lowered the production of hCTLA4Ig by 74%.

Discussion

siRNA has provided a powerful tool for sequence-specific gene silencing in plant and biomedical researches [25]. Double-stranded RNA ranging in length from 21 to 25 nt can cause RNAi, in which siRNA triggers the degradation of the target mRNA [30]. RNAi can be caused by various methods, such as long dsRNA, synthetic siRNA, plasmid-based short hairpin (sh) RNA vector, and virus-based shRNA vector [31]. Post-transcriptional gene silencing (PTGS) in plants is similar to RNAi in animals [34]. The mechanism of RNAi in synthetic siRNA occurs as follows. The chemically synthesized RNA duplex is delivered into the cells. Either the sense or antisense strand of the siRNA is incorporated into the RNA-induced silencing complex (RISC). According to the levels of homology between the siRNA and the target mRNA, the siRNA induces either the endonucleolytic cleavage of a specific target mRNA with high homology or the translational repression of a specific target mRNA with low homology [26].

The following requirements must be met in order to apply siRNA to plant cell suspension cultures: development of designed siRNA sequences, delivery of siRNA into the plant cells, and a detection system to measure the suppression of expression of the gene of interest [3]. After the 21-nt siRNA is deliberately designed with 3'-dTdT overhangs, the BLAST, which searches for regions of local similarity between sequences, must be performed to avoid nonspecific off-target gene silencing [23, 24]. The designed siRNA must then be effectively delivered into plant cells by the optimal delivery method.

Delivery methods for siRNA can be categorized as viral vector systems and nonviral vector systems. Viral vector systems include adenovirus and retrovirus systems, and as such, they are immunogenic, and their preparations are laborious and time consuming. Nonviral vector systems include lipoplexes (lipids+siRNA), polyplexes (polymers+siRNA), and lipopolyplexes (lipids+polymers+siRNA) [4]. One nonviral vector system that has been applied successfully is the transfection of siRNA into tobacco suspension cells by a cationic oligopeptide polyarginine–siRNA complex [29]. Polyethyleneimine (PEI) is a 25-kDa positively charged polymer that can combine with negatively charged nucleic acids to form siRNA complexes (siRNA+PEI) [7]. PEI-based polyplexes, the same used in the siRNA complexes in this study, represent another nonviral vector system that is nontoxic, stable, and easy to prepare compared with viral vectors [17]. The advantages of PEI-based polyplexes over other synthetic polymers are their high transfection efficiency and low cytotoxicity [33]. However, the transfection efficiency and low cytotoxicity of PEIs depend on the structure and type of PEI used [6]. PEIs are classified as branched PEI (bPEI) and linear PEI (lPEI). lPEI-based polyplexes provide higher transfection efficiency and cell viability than bPEI-based polyplexes [13]. The delivery of PEI-based polyplexes is associated with the endocytic pathway in plant cells. The endocytic machinery in plant cells includes coated vesicles, clathrin heavy chains, and the small ras-like rab of family GTPases, which are also involved in animal cell endocytosis [5]. PEI-based polyplexes can escape from the entrapment in the endosome by the proton sponge effect [4]. However, since plant cell walls are rigid and thick, effective delivery of PEI-based polyplexes into plant cells is still limited.

Sonoporation was therefore applied to enhance the efficiency of delivery of siRNA complexes into plant cells. However, since sonoporation can cause cell damage, the intensity of the ultrasound treatment must be optimized to maintain cell viability [15]. The optimum sonoporation treatment was determined as 40 kHz and 419 W for 90 s to transiently increase cell permeability without reducing cell viability, as shown in Fig. 2. The extent of siRNA complex delivery was estimated by fluorescence microscopy. The siRNA complexes were labeled with Cy3 to measure the delivery of siRNA complexes into plant cells using fluorescence microscopy. First, siRNA was labeled with Cy3 to avoid interference from PEI in the Cy3 labeling process. Next, Cy3-labeled siRNA was combined with PEI to form siRNA complexes. The fluorescence intensity of Cy3-labeled siRNA complexes was not diminished by conjugation with PEI, as shown in Fig. 3. In other studies, FM1-43 was used as a fluorescent endocytosis marker to image and quantify membrane endocytosis in intact plant cells [5], and siRNAs were delivered into transgenic rice, cotton, and pine cells by a nanosecond pulsed laser-induced stress wave [28].

In conclusion, PEI was used as a nonviral vector for exogenous gene delivery to transgenic rice suspension cells. PEI was combined with siRNA to form siRNA complexes. These PEI-based siRNA polyplexes were then delivered into plant cells. However, siRNA complexes could not sufficiently enter into cultured cells because of the nature of the plant cell walls. Sonoporation was therefore applied to improve the delivery of the siRNA complexes. As a result, the uptake of siRNA complexes was effectively facilitated by sonoporation. This investigation provides a simple and powerful method to deliver siRNA into cultured plant cells.

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