Supplementary Material

Optical Tweezers-Assisted siRNA Delivery for Gene Silencing in Plant Protoplasts

Yilun Liu [1], Pengpeng Zhou [1], Xinlin Chen [2] , Miao Peng [1,*], Guangzong Xiao [2,*], Hui Luo [2] and Yong Li [1]

[1] *Central South University of Forestry and Technology, Changsha, 410004, China*

[2] *National University of Defense Technology, Changsha, 410073, China.*

[3]*Nanhu Laser Laboratory, National University of Defense Technology, Changsha, 410073, Hunan, China*

** Correspondence author: pmiao913@163.com (Miao Peng); xiaoguangzong@nudt.edu.cn (Guangzong Xiao)*

NUMBER OF FIGURES: 1, NUMBER OF TABLES: 3, NUMBER OF PAGES: 8.

Preparation of Chemicals

Reagents and antibodies used in this study were obtained from commercial suppliers. Positively charged polystyrene (NH2-PS+, 5% solids) was purchased from Zhongke Leiming. CY3-labeled siRNA and all primers were obtained from Shanghai Bioengineering Co. and dissolved in ultrapure water before use. Cellulase and pectinase were from Coolaber; RT-qPCR reagents, PCR enzyme, and RNA reverse transcription kit were from Novagen; RNA extraction kit was from Accurate Biology.

Plant Growth

Transgenic tobacco seeds were provided by the laboratory of Huan Zhang at Jinan University[1]. Seeds were sown in 50- and 200-mm-diameter pots and grown in a controlled-environment chamber. Plants were maintained at 28 °C during the day and 22 °C at night, with 60% relative humidity (RH). At 4–6 weeks, healthy and undamaged leaves were selected for experiments.

Preparation of Protoplasts

Tobacco leaves (1 g) were cut into ~0.5 cm^2 pieces and immersed in an enzyme solution containing 0.1 g cellulase, 0.025 g pectinase, 0.4 M mannitol, and 8 mM CaCl2 (pH 5.5). The mixture was incubated at 40 rpm for 2 h at 26 °C in the dark. After enzyme treatment, the suspension was transferred to a centrifuge tube and centrifuged at 500 rpm for 5 min. The supernatant was discarded, and the protoplast pellet was washed three times with W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, and 5 mM glucose). Washed protoplasts were resuspended in MMM solution (15 mM MgCl2, 0.1% MES, and 0.5 M glycol).

Complexation of CY3-siRNA with PS

PS solution (100 mg/mL) was diluted to a series of concentrations and mixed with siRNA at a defined mass ratio. 0.1 M NaCl was added to bring the solution to the final volume. The PS/siRNA mixture was sonicated in a water bath for 10 min at room temperature to promote the formation of siRNA–PS complexes by electrostatic adsorption.

Nanoparticle Size and Zeta Potential Measurement

The particle size distribution and zeta potential of positively charged modified PS and siRNA–PS particles were measured with a Malvern Zetasizer Nano ZS90 to characterize their surface properties. PS and siRNA–PS suspensions were diluted with nuclease-free water, transferred to separate centrifuge tubes, sonicated in a water bath for 15 min, and analyzed using the same instrument.

Transmission electron microscopy (TEM) Characterization of materials

The morphology and size of positively charged modified PS and siRNA–PS particles were examined by TEM, and their elemental composition was analyzed by energy-dispersive X-ray spectroscopy (EDS). Samples were diluted, ultrasonicated, drop-cast onto TEM grids, and air-dried. TEM images were acquired first at low magnification to locate particles and then at higher magnification for morphological imaging. EDS analysis was performed in STEM mode.

Confocal laser scanning microscopy (CLSM) Characterization of materials

The fluorescence properties of positively charged modified PS and siRNA–PS particles and their silencing effect after delivery were examined by CLSM using a Zeiss Ti2-E inverted fluorescence microscope. For probe characterization, blue fluorescent PS microspheres and CY3-labeled siRNA were imaged separately using the blue channel (excitation 350–488 nm; emission 450–520 nm) and the CY3 channel (excitation 550 nm; emission 570 nm), respectively. For gene silencing, GFP fluorescence was imaged at excitation/emission wavelengths of 488/507 nm. Images were acquired using the instrument's default acquisition settings, without manual adjustment of laser power, gain, or pinhole. CY3-tagged siRNA–PS was tracked, and GFP fluorescence intensity was recorded. Images were analyzed with ImageJ to assess siRNA delivery and quantify fluorescence intensity.

Stability Testing of siRNA-PS Complex

Short-term stability at room temperature. The siRNA–PS probe suspension was incubated at room temperature for 0, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h.

At each time point, an aliquot was collected and analyzed by agarose gel electrophoresis at approximately 120 V for 30 min. The appearance of a free siRNA band indicated detectable dissociation of siRNA from the PS microspheres.

Thermal stability. Aliquots of the siRNA–PS probe suspension were incubated at 15, 20, 25, 30, 35, 40, 45, and 50 °C for 30 min. After incubation, the samples were analyzed by agarose gel electrophoresis under the same conditions. The appearance of a free siRNA band indicated partial dissociation of siRNA from the PS microspheres.

Resistance to RNase A digestion. Free siRNA (240 ng) and siRNA–PS complexes containing an equivalent amount of siRNA (240 ng) were separately incubated with 3 μL of RNase A solution (10 μg/mL) at 25 °C for 30 min. To release siRNA associated with the PS microspheres, a solution containing 2% SDS and 1.5 M NaCl was added, and the samples were incubated at 95 °C for 1 h. The released nucleic acids were then analyzed by agarose gel electrophoresis. A detectable siRNA band after RNase A treatment indicated protection from RNase A-mediated degradation, whereas its absence or marked reduction indicated degradation. Free siRNA was included as a control to assess its susceptibility to RNase A under the same conditions.

siRNA-PS gene silencing

Total RNA was extracted from protoplasts 24 h after delivery using an RNA extraction kit and reverse transcribed to complementary DNA (cDNA) with HiScript II reverse transcriptase. cDNA was analyzed by real-time quantitative PCR (qPCR). Primers were designed to amplify GFP and elongation factor 1 (EF1), and GFP expression was quantified using the comparative threshold cycle (ΔΔCt) method.

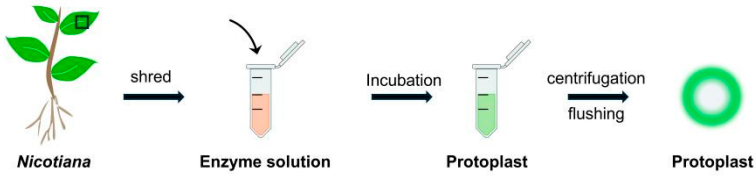


Figure. S1. Schematic diagram of protoplasmic system preparation process.

Table S1 siRNA sequence

Gene sequence name	Gene sequence
siRNA-GFP-sense	GGUGAUGCAACAUACGGAATT
siRNA-GFP-antisense	UUCCGUAUGUUGCAUCACCTT
non-specific siRNA -sense	UUCUCCGAACGUGUCACGUTT
non-specific siRNA -antisense	ACGUGACACGUUCGGAGAATT

Table S2. Zeta potential data before and after nucleic acid probe binding.

Zeta Potential classes (mV)	Intensity Distribution Data (counts)	
	PS	siRNA-PS
-40--30	0	8154.21579
-30--20	0	147551.5723
-20--10	0	104685.5723
-10-0	87381.96998	6535.458496
0-10	290666.1484	0
10-20	71231.45312	0

Table S3. Particle size data and PDI index before and after nucleic acid probe binding.

Group	Average size (nm)	PDI
PS(+)	538.4	0.556
siRNA-PS	542.8	0.601

1. Zhang, H.; Zhang, H.; Demirer, G.S.; González-Grandío, E.; Fan, C.; Landry, M.P. Engineering DNA Nanostructures for siRNA Delivery in Plants. *Nature Protocols* **2020**, *15*, 3064–3087, doi:10.1038/s41596-020-0370-0.