

Efficient delivery of small interfering RNA to plant cells by a nanosecond pulsed laser-induced stress wave for posttranscriptional gene silencing

Wei Tang^{a,*}, Douglas A. Weidner^b, Benjamin Y. Hu^c,
Ronald J. Newton^a, Xin-Hua Hu^d

^a Department of Biology, Howell Science Complex, East Carolina University, Greenville, NC 27858-4353, USA

^b Department of Microbiology and Immunology, East Carolina University School of Medicine, Greenville, NC 27858-4354, USA

^c North Carolina School of Science and Mathematics, 1219 Broad Street, Durham, NC 27715, USA

^d Department of Physics, Howell Science Complex, East Carolina University, Greenville, NC 27858-4353, USA

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Abstract

Small interfering RNA (siRNA) induced posttranscriptional gene silencing (PTGS) has been an efficient method for genetic and molecular analysis of certain developmental and physiological processes and represented a potential strategy for both controlling virus replication and developing therapeutic products. However, there are limitations for the methods currently used to deliver siRNA into cells. We report here, to our knowledge, the first efficient delivery of siRNA to plant cells by a nanosecond pulsed laser-induced stress wave (LISW) for posttranscriptional gene silencing. Using LISW, we are able to silence gene expression in cell cultures of three different plant species rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and slash pine (*Pinus elliottii* Engelm.). Gene silencing induced by siRNA has been confirmed by northern blot, laser scanning microscopy, and siRNA analysis. These data suggested that LISW-mediated siRNA delivery can be a reliable and effective method for inducing PTGS in cultured cells.

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Keywords: *Gossypium hirsutum*; Laser-induced stress wave; *Oryza sativa*; *Pinus elliottii*; Small interfering RNA

1. Introduction

Posttranscriptional gene silencing (PTGS) induced by small interfering RNA (siRNA) targeting a variety of endogenous genes or transgenes in cultured cells has recently emerged as a powerful technique that is capable of suppressing expression of individual genes with a high degree of specificity [1,2]. Since the first demonstration of siRNA induced gene silencing shown by effective repression of a luciferase reporter gene in mouse (*Mus musculus*) liver [3], siRNA has been delivered into different cells and tissues by various methods including viral vector mediated delivery [4,5], lipid-based delivery [6,7], atelocollagen mediated

delivery [8], microinjection [9], and feeding cells in siRNA containing medium [10] in animal systems.

In plant systems, PTGS has been studied by a number of methods of double-stranded RNA (dsRNA) or siRNA delivery including: (1) transforming plants with dsRNA-forming vectors for the selected gene by *Agrobacterium*-mediated transformation [11,12], (2) delivering cognate dsRNA of *uidA* (GUS) and TaGLP2a::GFP reporter genes into single epidermal cells of maize (*Zea mays*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) by particle bombardment [13], (3) introducing a tobacco rattle virus (TRV)-based vector into tomato (*Lycopersicon esculentum*) by infiltration [14], (4) delivering double-stranded RNA (dsRNA) into tobacco (*Nicotiana tabacum*) suspension cells by cationic oligopeptide polyarginine–siRNA complex [15], and (5) infecting plants with viral vectors that produce dsRNA [16]. In addition, *Agrobacterium*-mediated transient expression was successfully adopted to study PTGS in the intact tissues without generating

Abbreviations: GFP, green fluorescent protein; LISW, laser-induced stress wave; PTGS, posttranscriptional gene silencing; siRNA, small interfering RNA

* Corresponding author. Tel.: +1 252 328 2021; fax: +1 252 328 4178.

E-mail address: tangw@mail.ecu.edu (W. Tang).

transgenic plants [17]. Most of these methods require vector construction and plant transformation. In this study, we have developed a novel physical method using nanosecond pulsed laser-induced stress wave [18] to efficiently deliver siRNA into plant cells.

Nanosecond pulsed laser-induced stress wave (LISW) has been reported to deliver drugs to animal cells and tissues [18]. Recently, LISW as a new physical method has been reported to be efficient in delivering DNA fragments into rat (*Rattus* sp.) skin [19], murine fibroblast NIH 3T3 cells [20], and the mouse central nervous system [21], because of the high spatial controllability of the optical energy of a laser beam. Considering the chemical similarity of double stranded siRNA and small DNA fragment, we first used the nanosecond pulsed laser-induced stress wave as a functional genomics tool to deliver siRNA into cultured plant cells of rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and slash pine (*Pinus elliottii* Engelm.) for gene silencing.

2. Materials and methods

2.1. Preparation of transgenic cell lines

Transgenic cells of rice (*O. sativa* L.), cotton (*G. hirsutum* L.), and slash pine (*P. elliottii* Engelm.) were prepared as previously described [22,23] using *Agrobacterium tumefaciens* strain GV3850 carrying pBIN-*mgfp5-ER* [24,25] to transform callus cultures. After PCR, Southern blotting, northern blotting, cell growth, and green fluorescence analyses, three stable transformed cell lines each containing one copy of the pBIN-*mgfp5-ER* T-DNA and with similar growth rate and GFP expression were selected from rice (Ri), cotton (Co), and slash pine (Sp) and used for siRNA-mediated *gfp* silencing experiments.

2.2. siRNA synthesis and delivery

Twenty-one-nucleotide RNA with 3'-dTdT overhangs (Fig. 1) was synthesized by QIAGEN Inc. (Valencia, CA 91355, USA). Complementary double-stranded siRNAs with 2 nt overhangs were obtained by spontaneous annealing of mixtures of the antisense and sense oligoribonucleotides at 90 °C for 1 min and at room temperature for 1 h. For siRNA-

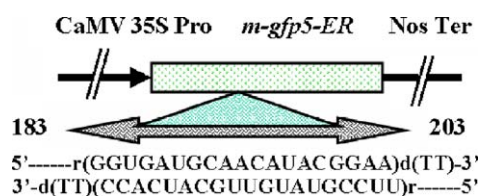


Fig. 1. Linear maps of *m-gfp5-ER* gene indicating the localization of the *m-gfp5-ER*, a modified GFP protein with an endoplasmic reticulum targeting sequence; *CaMV35Spro*, the cauliflower mosaic virus 35S promoter; *NosTer*, the terminator from nopaline synthase gene. Arrows indicate gene translation orientation. The probe used in northern blot analysis of transgenic cells is the 816 bp fragment of the *m-gfp5-ER* gene. The sequences of sense and antisense siRNA are indicated immediately below the *m-gfp5-ER* gene, and the position of siRNA is between nucleotides 183 and 203 of the *m-gfp5-ER* gene.

mediated *gfp* silencing experiments, a nanosecond pulsed laser-induced stress wave (LISW) was used to deliver siRNA to transgenic cells (Fig. 2). Three micrograms of siRNA per 3 ml cell cultures (300 mg of transgenic cells) were used and were delivered by using a nanosecond pulsed laser-induced stress wave (LISW).

The LISW was generated in a black rubber sheet of 0.6 mm in thickness by irradiating the rubber sheet with a single 12 ns laser pulse at a wavelength of 1064 nm and repetition rate of 5 Hz from a Q-switched Nd:YAG laser (Surelite I-10, Continuum). As shown in Fig. 2, the output laser beam was reduced in beam size and collimated with two lenses (L1 and L2) to a diameter of 5.0 mm before expanded with a diverging lens (L3) to illuminate the rubber sheet with a diameter of 27 mm on the rubber sheet. Due to the high irradiance of the incident nanosecond laser pulse and nearly 100% absorption, high-speed shock and transient acoustic waves are generated in the black rubber and lead to transient stress wave, as the result of optical breakdown [26]. The laser-induced stress wave has been studied for more than two decades [26,35]. When the irradiance on the black rubber exceeds the breakdown threshold, which is the case here, the pressure at the front of the generated shock wave and transient acoustic waves is determined by the total energy of the laser pulse and nearly independent of pulse duration [36]. The black rubber sheet was coupled to the bottom of a glass bottle holding the cultured cells with a thin layer of gel to reduce acoustic impedance mismatch. As the stress wave propagates into the cell culture for siRNA delivery. The laser pulse energy incident on the rubber sheet was varied between 180 and 130 mJ with a combination of a half-wave plate and a polarizing beam splitter, leading to a maximum fluence of 31 mJ/cm² or maximum irradiance of 2.6 × 10⁶ W/cm². For all pulse energies investigated, audible sound and flash were observed after application of each laser pulse which clearly indicates the occurrence of optical breakdown and subsequent LISW in the rubber [26].

Samples have been treated with LISW with 1–7 laser pulses using a single-shot control switch to investigate possible dependence on pulse number. When multiple pulses were used,

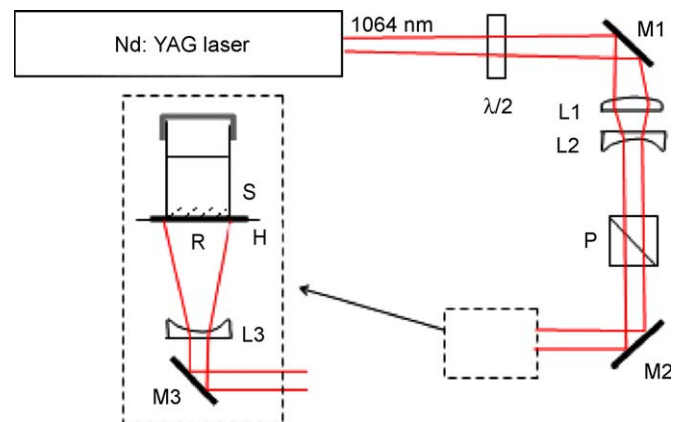


Fig. 2. The schematic of the nanosecond pulsed laser-induced stress wave experiment. $\lambda/2$: half-wave plate; M1/M2/M3: mirrors; L1/L2/L3: lenses; R: black rubber sheet; H: fixed bottle holder; S: cultured cell sample in a 20 ml bottle.

the sample was rotated between pulses to minimize the effect of non-uniform distribution of the energy within the beam profile. Just before laser irradiation, the suspension cell cultures were prepared freshly in bottles with bottom of 27 mm in diameter. All LISW treatments were carried out at the room temperature of 25 °C. After treatment, the cell cultures were returned to the incubator and samples were taken at different times for confocal microscopy and molecular analysis. Fig. 2 shows the experimental configuration of the LISW.

2.3. RNA isolation and northern blot analysis

Total RNA was isolated from 0.2 g transgenic cell cultures harvested through 42.5 µm filter papers and ground in liquid nitrogen using a RNeasy Mini Plant Kit (Germantown, MD 20874, USA) following the manufacturer's protocol. Five micrograms RNA was separated by agarose-gel electrophoresis. Electrophoresis and northern blotting of RNAs were performed as previously described [28]. Baked blots were pre-hybridized in 1 M NaCl, 1% SDS, 10% dextran sulphate and 50 µg/ml denatured herring sperm DNA at 64 °C, washed with 0.1× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 6.5), 0.5% SDS at 45 °C [28]. Digoxigenin (DIG)-Labelling *m-gfp5-ER* DNA (816 pb) (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA) was used as a hybridization probe. Equal loading of RNA samples was verified on the control of 25S rRNA. Hybridization signals were quantified with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and mRNA transcript level were normalized to rRNA level to overcome errors in RNA quantitation by spectrophotometry.

2.4. Laser scanning microscopy

To determine the effect of different culture times on *gfp* expression, cell cultures were observed at various times after treatments with a LSM 510 Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using excitation with the 488-nm Argon laser line and detection of emitted fluorescence between 500 and 520 nm. The confocal images of *m-gfp5-ER* expressed cells were created in the Expert Mode. Background correction was applied by adjusting fluorescence levels in a neighboring non-transgenic cell into zero. Thirty to 50 cells were used for each sample.

2.5. Detection of siRNA by RNase protection assay

Total RNA was extracted from transgenic cells with TRI reagent as described [27,28]. 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 [28] and was used for the RNase protection assay [29]. In brief, the enriched small RNA was hybridized to digoxigenin (DIG)-labelling *m-gfp5-ER* DNA (816 pb) (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA) probe overnight at 35 °C. siRNA in the enriched fraction binds to the probe during this period. The un-hybridized single-

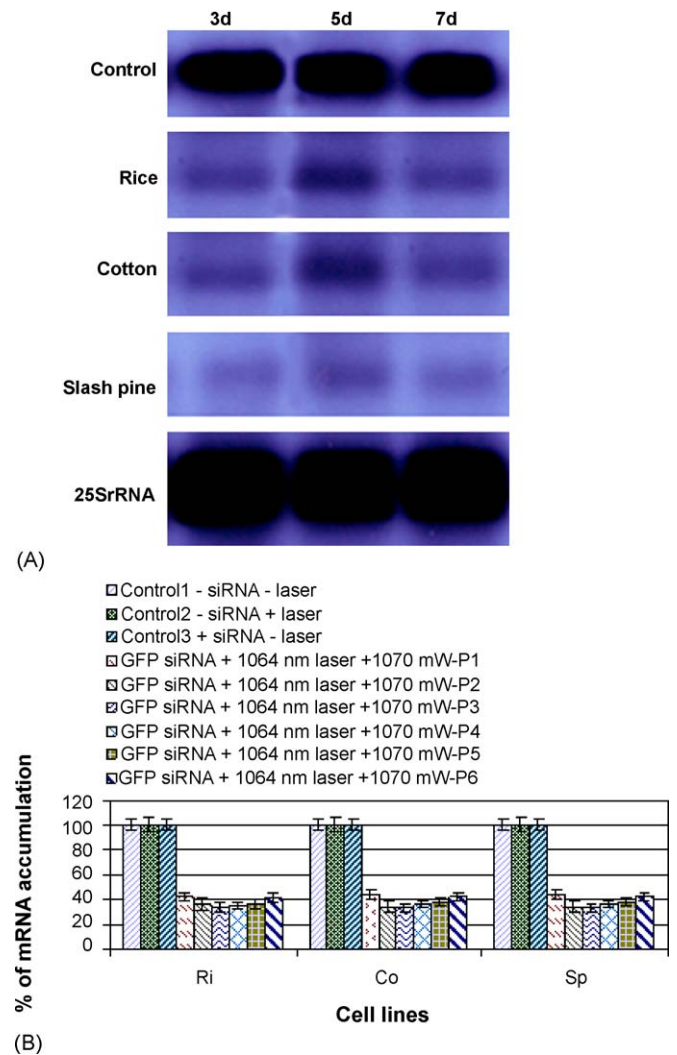


Fig. 3. Northern blot analysis of total RNA from transgenic cells. RNA (5 µg) was extracted from transgenic cells of cotton (*Gossypium hirsutum* L.), rice (*Oryza sativa* L.), and slash pine (*Pinus elliottii* Engelm.) at 3, 5, and 7 days after treatment with siRNAs, and were hybridized (at 65 °C) with the 816-bp *m-gfp5-ER* probe corresponding to the *m-gfp5-ER* gene, which was labeled with DIG (A). The control panel is *gfp* transgenic slash pine cells (Sp) that were not treated with siRNA. The integrity and the amount of RNA applied to each lane were verified by the control of tobacco 25SrRNA (lower panel). (B) Hybridization signals were quantified with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and mRNA transcript level were normalized to rRNA level to overcome errors in RNA quantitation by spectrophotometry. Percentage of mRNA accumulation in silenced and control cells of cell lines Ri, Co, and Sp 5 days after treatment. Values represent the means ± S.E. Times of 1064 nm laser pulses (1–6 times, P1–P6) at power of 1070 mW did not increase the degree of gene silencing.

stranded RNA was digested with 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 and followed by fractionation on a 15% denaturing PAGE gel and exposure to X-Omat AR-2 film (Fisher Scientific) for 15 min. In vitro-prepared small 21-nucleotide antisense RNA was used as the marker.

2.6. Statistical analysis

All experiments were repeated three times. Data obtained from different experiments were analyzed by using the General Linear Model procedure of SAS (SAS, Cary, NC), employing ANOVA models. The significant differences between mean values obtained from three independent experiments were made with the least significant difference test at 5% level of probability. Each value was presented as means plus standard errors of the mean.

3. Results

3.1. Production of transgenic cells

Transgenic cells of rice (*O. sativa* L.), cotton (*G. hirsutum* L.), and slash pine (*P. elliottii* Engelm.) with insertion of sense *m-gfp5-ER* reporter gene (Fig. 1) were produced using *A.*

tumefaciens (Strain GV3850) mediated gene transfer as described in Tang et al. [22]. After PCR and Southern blotting analysis (data not shown), 49 transgenic cell lines with one copy of *m-gfp5-ER* were selected from each species. After northern blotting and cell growth rate analysis (data not shown) of transgenic cell lines with one copy of *m-gfp5-ER*, three transgenic cell lines Ri (rice), Co (cotton), and Sp (slash pine) were selected and used for siRNA-mediated *gfp* silencing. Transgenic cell lines Co, Ri, and Sp were sub-cultured weekly for 6 weeks to multiply the cell cultures in an incubator shaker at 150 rpm and 25 °C.

3.2. Delivery of siRNA to cultured cells by a nanosecond pulsed laser-induced stress wave

For experiments of LISW-mediated siRNA delivery, suspension cells were re-suspended in fresh liquid medium. Freshly prepared suspension cells were distributed into experimental

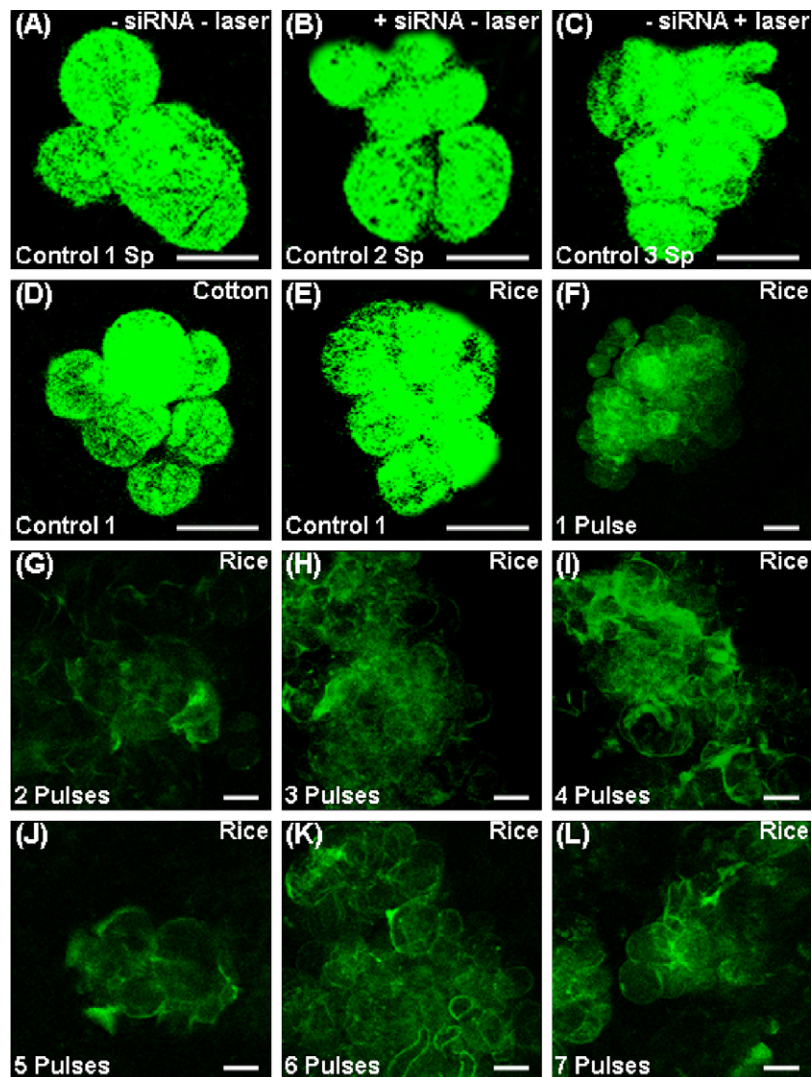


Fig. 4. Laser scanning microscopy of silenced GFP expression by siRNA in transgenic cells of rice (F–L), cotton (D), and slash pine (Sp, A–C) 1 week after treatment with siRNA. Transgenic cells without treatment of both siRNA and laser (A, D, and E), transgenic cells treated with siRNA but not laser (B), and transgenic cells treated with laser but not siRNA (C) were used as controls. GFP fluorescence was decreased in all transgenic cells treated with siRNA and laser (pulse time 1–7, rice cells were used as an example) (F–L) (bars = 50 μ m).

bottles for laser irradiation (the detail is described in Section 2). RNA-blot hybridization was used to compare the degradation of *gfp* mRNAs in silencing transgenic cells (Ri, Co, and Sp) 3, 5, and 7 days after LISW-mediated siRNA delivery. Fig. 3A shows that silencing of *gfp* expression was correlated with a dramatic decrease in *gfp* mRNA accumulation in three transgenic cell lines. The *gfp* transgenic cells treated with LISW but no siRNA, or treated with siRNA but no LISW demonstrated no decrease in mRNA levels (Fig. 3B), which has the same levels of mRNA from transgenic cells not treated with siRNA and LISW. The same silencing signature was observed in all three transgenic cell lines. These results confirm that the LISW-delivered siRNA has efficiently induced *gfp* silencing at the posttranscriptional level. Gene silencing was observed only after transgenic cells were treated with LISW and siRNA. Influence of laser pulse number on gene silencing demonstrated that the mRNA transcripts of the *m-gfp5-ER* were decreased when one to seven laser pulses were used (Fig. 3B). No differences in decreased level of mRNA transcripts were observed when two to seven laser pulses were used (Fig. 3B).

3.3. Laser scanning microscopy of *gfp* silencing

The silencing of *gfp* expression was monitored with help of a LSM 510 Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using excitation with the 488 nm Argon laser line and detection of emitted light between 500 and 520 nm in transgenic cells over a 3 weeks culture period. Gene silencing was observed in transgenic living cells 12 h after LISW-mediated siRNA delivery (data not shown). High-level silencing (decreasing of 80–95% of *gfp* transcripts) of *gfp* expression was observed on 3–7 days after LISW-mediated siRNA delivery. Stable gene silencing (more than 2 months) was observed 5 days after LISW-mediated siRNA delivery in three transgenic cell lines Ri, Co, and Sp. Fig. 4 demonstrates changes of green fluorescence in transgenic living cells 1 week after LISW-mediated siRNA delivery in three transgenic cell lines. No silencing was detected from transgenic cells treated with siRNA but not LISW or treated with LISW but not siRNA.

These results confirm the efficiency of gene silencing induced by LISW-mediated siRNA delivery. Thus, it appears that the method described here provides a reliable approach for analyses of gene silencing. To assess the effectiveness of LISW-delivered siRNA-mediated *gfp* silencing in plant cell systems, we monitored *gfp* silencing over 3 weeks after LISW-mediated siRNA delivery by confocal images and northern blotting. These results demonstrated the method presented here could be useful in study of gene silencing using LISW-mediated siRNA delivery and using GFP as a visual marker.

3.4. Detection of siRNA in *gfp* transgenic cells

To further investigate gene silencing induced by LISW-mediated siRNA delivery, we 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 in cells treated with siRNA and not in the

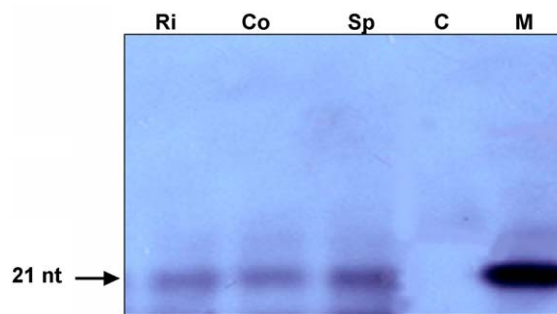


Fig. 5. Detection of small RNAs. Low molecular weight RNA fractions were isolated from transgenic cells of rice (Ri), cotton (Co), and slash pine (Sp) separated on polyacrylamide gels, blotted onto Hybond N⁺ membranes, and hybridized with 816 bp *gfp*-coding sequences. The 21 nt siRNA oligomers were used as size controls (size indicated in nucleotides). Each lane contains the low molecular weight RNA fraction of transgenic cells treated with siRNA. No specific signal of siRNA could be detected in transgenic cells without treatment of siRNAs with the probes. Line M, The 21-nucleotide small *gfp*-specific RNAs were as a marker. Line C, *gfp* transgenic cells without treatment with siRNA was presented as a control. Lines Ri, Co, and Sp, transgenic cells of rice, cotton, and slash pine 1 week after treatment with siRNA gave rise to small *gfp*-specific RNAs of approximately 21 nucleotides.

control (Fig. 5). We have demonstrated that LISW delivered siRNA effectively and conveniently induced PTGS in rice cotton and slash pine suspension cells.

4. Discussion

Gene silencing of endogenous or reporter genes have been described in animal cells and transgenic plant cells [1,30–32]. In cases of siRNA-mediated gene inactivation, it was reported that gene silencing can be stable, inducible, and reversible [32], and this kind of regulation of RNA interference has broad applications in e.g. the areas of mammalian genetics, molecular therapeutics, and plant virus control [33,34]. The *gfp* gene does not exist in plant cells, the use of *gfp* transgenic cells as material to analyze the degree of siRNA-mediated *gfp* silencing is an ideal choice for investigation of gene silencing, because off-target effect can mostly be excluded in such a system [27]. In this study, we have analyzed the effectiveness of PTGS among three *gfp* transgenic cell lines including monocots (rice), dicots (cotton), and gymnosperm (slash pine) systematically by identifying RNAi lines that each carries only a single-copy of the transgene and by LISW-mediated siRNA delivery.

Although different methods of siRNA delivery have been reported in animal systems [3–10] and in plant systems [11–16], most of these methods require vector construction and plant transformation. Some of them are with some problems such as assurance of safety, the immune response to viral vectors, low transfer efficiency, serious side effects, and limited targeting characteristics. In the present study, we developed a novel LISW-mediated siRNA delivery method for PTGS in cultured plant cells. Because of the high spatial controllability of laser energy, this method may be suitable for not only plant system, but also animal system. With this method, only a few single laser pulses were needed for successful delivery of siRNA to cultured cells and the efficiency obtained was

comparable to or higher than that obtained with particle bombardment [27]. With particle bombardment, 75–90% of *gfp* silencing was obtained. With the LISW-mediated siRNA delivery method, 80–95% of *gfp* silencing was obtained. The mechanism of LISW-mediated siRNA delivery through the plasma membrane is not clear, but the probable functions of LISW include denaturation of membrane proteins, membrane disruption by cavitation, transient disruption of the plasma membrane by shear force, and acceleration of exogenous molecules [19–21].

Generation of shock waves and transient acoustic waves, collectively noted as stress wave here, in condensed matter by short laser pulse are caused by optical breakdown. When the irradiance on the black rubber exceeds the breakdown threshold, which is the case here, the pressure at the front of the generated shock wave and transient acoustic waves is determined by the total energy of the laser pulse and nearly independent of pulse duration [36]. Using a standard model of laser induced stress wave and results obtained in water and cornea [26,35], we estimated that the maximum pressure reaching the cultured cells at a distance of about 2 mm from the air-rubber interface is about 700 bar and speed of travel is between 1500 and 2500 m/s. Our estimated pressure agrees with the reported values measured with a hydrophone [19]. Furthermore, the activation time of the stress wave on the cells in the aqueous culture medium, before fully resolved by energy dissipation, is estimated to be less than 0.1 s. Therefore, our results reported here indicate the LISW-mediated siRNA delivery can be accomplished with one single pulse of energy 130 mJ or larger. Even though the underlying mechanism is not fully understood, it is plausible that the stress wave with high-pressure front induces various channels for the penetration of siRNA carrying particles through the cell wall and membrane. We note here that the Q-switched Nd:YAG laser used in this study for generation of nanosecond pulses at 1064 nm is a commercial system that is readily available with compact size, high reliability and reasonable cost (US\$ 15,000/unit). Nominal lifetime of the pumping lamp used in this type of lasers is about 25 million pulses. Therefore, the maintaining cost of laser-induced gene transfer is much less than other physical techniques such as particle bombardment after the initial purchase of the laser because no other agents are needed for laser treatments.

In the present investigation, we have investigated the LISW-delivered siRNA-mediated PTGS in three transgenic cell lines and our results showed consistent changes in the levels of *gfp* silencing. We found that RNAi lines targeting the same gene generally reduced target transcript levels to a similar extent. In addition, this study illustrates the usefulness of siRNA delivery using nanosecond pulsed laser-induced stress wave for gene silencing in both angiosperms and gymnosperm species, and highlights the potential of LISW-mediated siRNA delivery for effective gene silencing in cultured plant cells. We rationalized that similar levels of PTGS are due to the effective LISW-mediated siRNA delivery. In three transgenic cell lines, the siRNA reproduced the same effects, indicating that this was not due to a synthesis error or contaminant. Together, our data

suggest that siRNA-mediated PTGS can be achieved with the LISW-mediated siRNA delivery. In view of convenience of preparation and delivery of siRNA, LISW-delivered siRNA-mediated PTGS technology described in this investigation can be an efficient functional genomics tool and may be applied to the studies of gene function or some other molecular genetic researches. To our knowledge, our results present the first evidence that PTGS may be induced by the LISW-mediated siRNA delivery.

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