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Oligopeptide polyarginine-mediated delivery of short interfering RNA to cells for gene silencing

***Wei Tang*[1,2*]*; Yongjun Fei*[1]*; Michael Page*[2]**

[1]*College of Horticulture and Gardening, Yangtze University, China*

[2]*Institute for Genome Sciences and Policy, Duke University, USA*

***Corresponding Author(s): Wei Tang**

College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, China

Tel: +86-13921074062, Fax: +86-716-8066262

Email: wt10yu604@gmail.com

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Abstract

Short interfering RNA (siRNA) mediated gene silencing plays an important role in functional identification of novel genes and therapeutic product development. Double stranded siRNA molecules are usually delivered to animal cells by injection and to plant cells by particle bombardment. Here, we reported that oligopeptide polyarginine (POA)/siRNA complex can be efficiently delivered to cultured cells for gene silencing in four plant species. The similar levels of the short interfering RNA-mediated *gfp* silencing were observed in four transgenic plant cell lines. Experimental results of northern blotting analysis and laser scanning microscopic images, as well as mRNA expression of siRNA analysis confirmed that POA-mediated delivery of siRNA has reduced the level of *gfp* expression and caused GFO silencing. These results suggested that POA-mediated delivery of siRNAs could be an effective method for gene silencing in plant cells.

Keywords: Gene silencing; Oligopeptide polyarginine; Plant cell; Pinusvirginiana; Short interfering RNA

Abbreviations: GFP: Green fluorescent protein; POA: Oligopeptide polyarginine; 35S: Cauliflower mosaic virus 35S promoter; siRNA: Short interfering RNA.

Introduction

Gene silencing induced by short interfering RNAs (siRNAs) has been an important and a powerful technique that is able to reduce expression of targeted individual genes with a high degree of specificity [1]. Gene silencing mediated by siRNA has proved to be an efficient method to investigate gene function in different research fields in functional genomics [2,3]. It has been reported that gene silencing can be abrogated by a single mismatch between a siRNA and its target mRNA [4]. The siRNAs has been reported to play a central role in triggering mRNA degradation of their target genes [4,5]. Gene silencing induced by siRNAs has been reported in different organisms, such as, *Drosophila* [6], nematodes [7,8], trypanosomes [9], *Nicotianabenthamiana* [10], and *Nicotianatabacum* [11]. In mice, siRNA can effectively silence *Fas* gene and protect mice against renal ischemia–reperfusion injury [12]. In tobacco BY2 protoplasts, siRNA blocked the mRNA accumulation of replication-associated protein (AC1) of the Gemini virus African Cassava Mosaic Virus (ACMV) from Cameroon by up to 91% and inhibited accumulation of the ACMV genomic DNA by about 66% [11].

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Gene silencing-mediated by siRNAs could be an efficient experimental tool to investigate the cellular pathways in different cell lines and to identify functions of novel genes in different plant and animal species [13]. Since the siRNA expression plasmids were first delivered into mouse liver by hydrodynamic transfection to effectively repress expression of a luciferase reporter gene [14,15], various methods have been 1 used to deliver siRNA in to target tissues and cells. These methods include viral vector-mediated delivery [16,17], lipid-based delivery [18,19], siRNA injection [12], and atelocollagen-meditaed siRNA transfection [13]. In plant species, both protoplast transfection [11] and particle bombardment [10,20] have been successfully used to deliver siRNA to target cells. However, most of these methods require vector construction and expensive experimental facilities. Functional studies and reports of RNAi-delivery strategies for cells of different plant species are very limited.

Oligopeptide Polyarginine (POA) has been used as a tool for intracellular delivery of biologically active proteins and DNA through in vivo protein transduction [21]. The human immunodeciency virus-tat transcription factor can be used to form the Protein Transduction Domain (PTD) of POA that is able to mediate delivery of dsRNA for gene silencing in plant cells [21,22]. The protein transduction domain in POA contains cationic arginine or lysine that may play a critical role in transverse cell membrane [22,23]. It has been reported that cationic oligopeptide β-galactosidase polypeptide fused to a PTD sequence is able to deliver to molecules after intraperitoneal injection of mice [22,24]. PTD can interact with nucleic acids in cells and form complex to be delivered into different mammalian cells [22,25,26]. Based on the fact that both double stranded siRNA and DNA are negatively charged molecules, we use a synthetic cationichomoarginine oligopeptide (12-mer) POA as a tool and use GFP as a reporter to deliver siRNA into cells of different plant species to induce gene silencing.

In the present investigation, we have examined the function of a synthetic cationic homoarginine oligopeptide for delivery of siRNA in *gfp* transgenic cells of different plant species, including cotton (*Gossypiumhirsutum* L.), rice (*Oryza sativa* L. cv. Radon), Virginia pine (*Pinusvirginiana* Mill.), and eastern white pine (*Pinusstrobus* L.). Our results demonstrated that oligopeptide polyarginine (POA)/siRNA complex can be efficiently delivered to cultured cells in four plant species. Our study based on POA-mediated delivery of siRNAs demonstrates the success of siRNA-mediated gene silencing in both angiosperm and gymnosperm species, indicating cationic oligopeptide-mediated delivery of siRNA can be a novel method for gene silencing in cell cultures for physiological and biochemical investigations in plant cells.

Materials and methods

Generation of transgenic cell lines

Transgenic cells of cotton (*Gossypiumhirsutum* L.), rice (*Oryza sativa* L. cv. Radon), Virginia pine (*Pinusvirginiana* Mill.), and eastern white pine (*Pinusstrobus* L.) were generated as described in Tang et al. [27]. Cell suspension cultures of four plant species were transformed using the method of *Agrobacterium tumefaciens*–mediated transformation (strain GV3850) and the expression vector is pBIN-*m-gfp5-ER* [28,29]. After inoculation of cells, *Agrobacterium* was removed with 500mg/lTimentin (ticarcillin/clavulanic acid 3:0.1, SmithKline Beecham, Philadelphia, PA). TE liquid culture medium was use to obtain large quantities of transformed cell cultures [30]. After molecular analysis of PCR, Southern blotting, northern blotting, and green fluorescence analyses, we select four stable transformed cell lines (each containing one copy of the pBIN-*m-gfp5-ER* T-DNA) from Cotton (Co), Rice (Ri), Virginia pine (Vp), and eastern White pine (Wp), respectively, for POA delivery of siRNAs for gene silencing experiments.

Short interfering RNAs (siRNAs) preparation

The *gfp* siRNA (twenty-one-nucleotide RNA with 3'-dTdT overhangs, Figure 1) was synthesized by QIAGEN Inc. (Valencia, CA 91355, USA). The siRNA sequences were: sense strand 5'-r(GGUGAUGCAACAUACGGAA) d(TT)-3' (nucleotides 183-203) and antisense strand 3'-d(TT) (CCACUACGUUGUAUGCCUU) r-5' (Figure 1). The double-stranded siRNAs were generated by spontaneous annealing of the antisense and sense oligoribonucleotides at 90°C for 1 min and at room temperature for 1h. Oligopeptide Polyarginine (POA) was used to deliver siRNA to transgenic suspension cells of different species according to the procedure previously described [22]. The siRNA control is the *PtaAGP6* (GenBank No. AF101785) gene siRNA [sense strand 5'-r(UCAGCCGUAAUGCACGAUAGCG) d(TT)-3' (nucleotides 591–611) and antisense strand 3'-(TT) (AUGCCGUAGACUAGCAGUACCU)r-5', QIAGEN Inc. Valencia, CA 91355, USA].

Oligopeptide polyarginine (POA) synthesis and formation of siRNA/POA complex

The molecules of Oligopeptide Polyarginine (POA) were synthesized from Invitrogen (Carlsbad, CA 92008, USA). The optimal binding ratio of siRNA to POA was determined as previously described [22]. In brief, 90ng of *gfp* siRNA was mixed with 5,10,15,20,30,40, and 50ng) in PBS buffer (total volume of 10µl) and incubated at 4°C for 30min and electrophoresed in 1.8% agarose gel [22]. The optimum concentration of oligopeptide polyarginine for *gfp* siRNA was determined according to Unnamalai et al. (2004).

Delivery of *gfp*siRNA through POA complex to plant suspension cells

The *gfp* siRNAs at 5,10, and 30µg were mixed with oligopeptide polyarginine and the siRNA/POA complex was delivered to the transgenic suspension cells of four plant species. Five ml of 3-day-oldsuspension cells were rinsed with PBS to remove TE medium. After cells (1g fresh cells) were sonicated for 60 sec in sonicator (model PC5, L&R Manufacturing Co, Kearny, N.J.), the siRNA/POA complex were mixed with suspension cells in 500µl PBS. Cells that were not sonicated were used as controls. Gene silencing experiments of the delivery of *gfp* siRNA through POA complex were conducted three times. The viability of the treated cells was measured according to the method described by Unnamalai et al. (2004).

RNA isolation and northern blot analysis

Total RNA was extracted from transgenic cells (1.5g) of cotton (*Gossypiumhirsutum*L.), rice (*Oryza sativa* L. cv. Radon), Virginia pine (*Pinusvirginiana* Mill.), and eastern white pine (*Pinusstrobus*L.) using a RN easy Mini Plant Kit (Valencia, CA91355, USA). Electrophoresis and northern blotting of RNAs (10µg) were performed as described by Tang et al. [27,30]. The hybridization probe is the Digoxigenin (DIG)-Labellin g*m-gfp5-ER* DNA (816pb) (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA). The loading control is the tobacco 25S rRNA. Quantification of the hybridization signals was done with a Storm 860 Phosphor Imager (Molecular Dynamics,

Sunnyvale, CA).

Scanning electron microscopy

Scanning Electron Microscopy (SEM) was used to observe the morphological change of suspension cells of four plant species at different growth stages after cells were rinsed in distilled water for 30 min and dried on filter paper at 25°C for 30min, as previously described by Tang et al. [30] with some modifications. Samples were fixed in 2.5% glutaraldehyde in 0.1M PBS, dehydrated in ethanol, and washed in amyl acetate. After critical point dried under CO2, samples were examined directly and photographed using a Quanta200 environmental scanning electron microscope (FEI, Hillsboro, Ore.) at low vacuum mode, as previously described by Tang et al. [30].

Confocal laser scanning microscopy

LSM 510 Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY, USA) was used for taking images and for quantitative fluorescence determinations of GFP activity (excitation at488-nm, emitted light between 500 and 520 nm was applied to capture images). Images of GFP expressed plant cells of four species were created in the Expert Mode as previously described by Tang et al. [30]. Fluorescence intensities of GFP expressed plant cells of four species were calculated with the Zeiss LSM Image Examiner software (30 to 50 cells were used for each sample), as previously described by Tang et al. [30].

Detection of siRNA by RNase protection assay

Total RNA was isolated from transgenic GFP expressed plant cells of four species with TRI reagent as described [31,32]. Total RNA of GFP expressed plant cells of four species was precipitated with yeast RNA and ethanol dissolved in DEPC-treated water as previously described [31]. The small size RNA was enriched [31] before it was used for the RN aseprotection assay, as previously described [32].

Results

Efficient delivery of siRNA/POA complex and siRNA-mediated *gfp* silencing

Transgenic cells of Rice (Ri), Cotton (Co), Virginia pine (Vp), and eastern White pine (Wp)with one copy of T-DNA insertion of sense *m-gfp5-ER* reporter gene (Figure 1A) were produced using *Agrobacterium tumefaciens* (Strain GV3850) mediated gene transfer as described in Tang et al. (2005a). After PCR and Southern blotting analysis (data not shown), transgenic cells with one copy of *m-gfp5-ER* were selected and used for siRNA mediated *gfp* silencing. The optimum amount of the POA needed for interacting siRNA was determined as previously described Unnamalai et al. (2004). The amount of 5,10,15,20,30,40, and 50ng POA were mixed with 90ng siRNA to form a complex, respectively. When 90ng of siRNA and 30ng of POA were mixed, EtBrstaining indicated that siRNA was protected by POA (data not shown). For siRNA/POA mediated siRNA delivery in plant cells, we increased the concentrations of siRNA and POA. Our results demonstrated that siRNA can be effectively delivered to cultured cells by mixing 30µg siRNA, 10µg POA, and 1g fresh weight of cells (data not shown). When 10µg POA was used, the viability of treated cells was not changed (data notshown).

Northern blot analysis of total RNA from *gfp* transgenic plant cell lines of Ri, Co, Vp, andWp, at 15h after treatment with siRNA/POA complex, demonstrated that siRNA delivered by siRNA/POA complex inhibits *gfp* mRNA accumulation in all four transgenic cell lines (Figure 1B). There is no decrease of *gfp* mRNA in transgenic cells that were not treated with siRNA, or treated with sonication but not siRNA, or treated with POA but not siRNA, or treated with siRNA but not sonication and not POA. After transgenic cells were sonicated, percentage of mRNA accumulation in silenced cells decreased more than non-treated cells in cell lines Ri, Co, Vp, and Wp 5 d after different treatment (Figure 1C).

Enhanced delivery of siRNA/POA complex by sonication in transgenic cells

To investigate the role of sonication in siRNA/POA complex mediated siRNA delivery, we have analyzed the distribution of siRNA delivered with POA in four cell lines. Scanning electron microscopy of transgenic cells (Vp) demonstrated that there is no cell damage in cells that were not sonicated (Figure 2A). Scanning electron microscopy of transgenic cells (Vp) demonstrated that there is cell damage in cells that were not sonicated (Figure 2B). Cell damage derived from sonication may facilitate the delivery of siRNA into cells. However, analysis on distribution of siRNA delivered with POA in four cell lines demonstrated that POA can efficiently deliver not only *gfp* siRNA, but also aPtaAGP6 siRNA (Figure 2C). Our results on siRNA distribution in transgenic cell lines Ri, Co, Vp, and Wp showed that sonication results in double amount of siRNA in four cell lines 6h after treatment with siRNA (Figure 2D). There is no siRNA was observed in transgenic cell lines Ri, Co, Vp, and Wp 6h after treatment with siRNA but without sonication and POA, or with POA but without siRNA, or with sonication but without siRNA (Figure 2D).

siRNA delivered by siRNA/POA complex efficiently inhibits GFP expression

The efficiency of gene silencing mediated by the siRNA delivery through siRNA/POA complex was quantitatively determined from the confocal images of GFP expressed plant cells of four species taken by a LSM 510 Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY, USA). Figure 3 (A) shows changes of green fluorescence in transgenic living cells of GFP expressed plant cells, 15 h after treatment with siRNAs in transgenic cell lines Ri, Co, Vp, and Wp. GFP fluorescence was reduced more in all transgenic cells after sonicated and treated with siRNA/POA complex than sonicated or treated with siRNA/POA complex only. Figure 3 (B) demonstrates that the changes of gene silencing expressed by green fluorescence intensities in transgenic GFP expressed plant cells in transgenic cell lines Ri, Co, Vp, and Wp 15h after treated with siRNA. A higher (2-3 times) incidence of silencing was detected 15h in transgenic cells treated with sonication and siRNA/POA complex than siRNA/POA complex only (Figure 3B). No silencing was detected from transgenic cells GFP expressed plant cells of four species without the treatment of siRNA/POA complex and non-transgenic cells.

Formation of siRNA in *gfp* transgenic cells

We further substantiated gene silencing of GFP expressed plant cells of four species by showing the presence of siRNA in GFP expressed plant cells of four species using RNase protection assay. The *gfp* siRNA, corresponding to the 21-nt region in the *gfp* gene, was detected only in cells of GFP expressed plant cells of four species treated with siRNA/POA complex and not in the control (Figure 4A and B). These results demonstrated that siRNA/POA was effectively and conveniently delivered to cultured cells to induce gene silencing in plant cells. Results from

siRNA analysis demonstrated that the amount of siRNA in plant cells increased 3-14 d after treatment of siRNA/POA complex (Figure 4C). During 14-21 d after treatment of siRNA/POA complex, siRNA accumulation did not increase significantly (Figure 4C). Based on the convenience of preparation and delivery of siRNA/POA complex, delivery of siRNA/POA complex may be a effective method for gene silencing technology that may be useful to the studies of functional genomics of different plant species or some other cell physiological and biotechnological researches.

Discussion

It has been reported that gene silencing in plant cells of different species mediated by short interfering RNA (siRNA) can be stable, inducible, and reversible and it has very important applications in molecular therapeutics of human diseases [5,33,34]. However, application of RNAi as a useful therapeutic method requires an efficient delivery method [4,13,35]. Oligopeptide Polyarginine (POA) has been reported for DNA delivery in animal cells [22]. However, POA mediated delivery of siRNA has not been reported in any plant studies. The *gfp* gene does not exist in plant cells and can be useful as a marker to analyze the degree of siRNA-mediated *gfp* silencing, because off-target effects was excluded in such a system [20]. It has been reported that phenotypic changes derived from strong RNAi effects were correlated with expression levels of the targeted genes in *Caenorhabditiselegans*, [7,36-38].

RNAi effectiveness can be effected by the composition of genomic DNA sequence, patterns of spatial and temporal gene expression, and the turnover rate of the normal RNA of targeted genes in a gene specific manner [3,4]. However, it has been reported that the obstacle to develop the RNAi technology-based therapeutic products required a suitable method of the siRNA delivery in cells [13]. Although, viral delivery systems are efficient, the serious side effects can be concerns of the method [13]. Lipid delivery systems can be effective but induce immune activation *in vivo* [13]. Heidel*et al.* [39] showed that synthetic siRNAs can be delivered to mice cells for reducing expression of exogenous target genes. Takeshita et al. (2005) has established an efficient atelocollagen-based method of siRNA delivery to study bone-metastatic tumors. Their method can reduce the off-target effect of siRNA [13].

Currently, we do not succeed in using the atelocollagen-based siRNA delivery method for siRNA-mediated gene silencing in cultured plant cells. We have investigated the specificity of siRNA-mediated RNAi in transgenic cell lines of four plant species that demonstrated the consistent changes in the levels of *gfp* gene silencing. Our results demonstrated that POA-mediated delivery of siRNA resulted in gene silencing in four transgenic cell lines. This method can be used to investigate the function of target gene by degrading the expression level of corresponding mRNA. POA-mediated delivery of siRNA can be an effective alternative to the widely used approaches such as T-DNA in sertional mutagenesis in different plant species for post-transcriptional gene silencing.

Based on our investigation, it is convenient to prepare and deliver siRNA into cells of different plant species, using siRNA/POA-mediated gene silencing technology. This method may be useful to the studies of gene function and signal transduction pathways. Our experimental results indicate that siRNA/POA complex can be used to deliver siRNA to cultured cells of different plant species. Furthermore, we have showed that POA-mediated siRNA delivery system is effective in four species. In conclusion, a technique to efficiently deliver siRNA to cultured plant cells in different plant species has been developed to inhibit specific target gene expression. To our best knowledge, it is the first evidence that delivery of siRNA/POA complexes in cultured angiosperm and gymnosperm cells may have potential in functional identification of novel genes in plant.

Figures

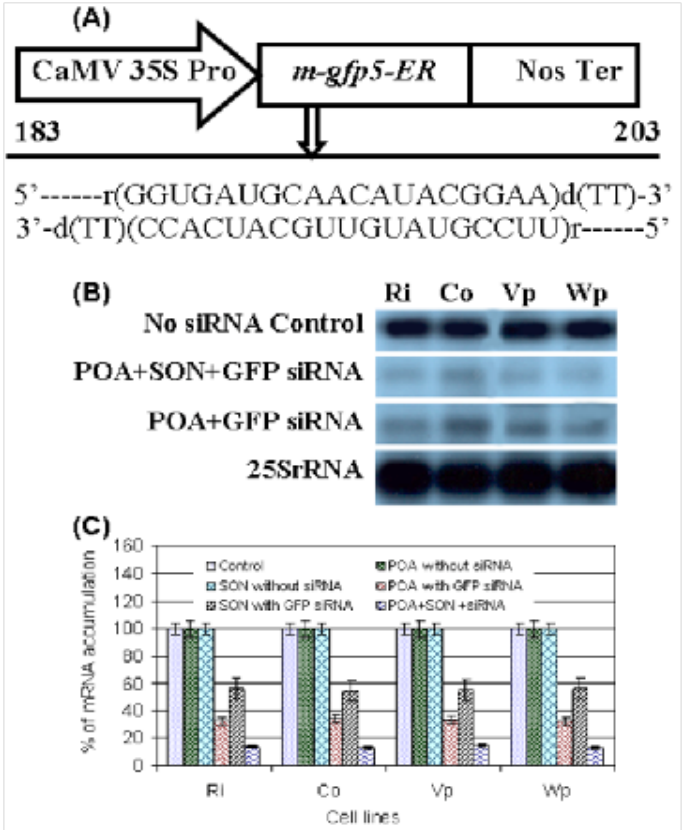

Figure 1: (A) Plasmid construction indicating the localization of the *m-gfp5-ER,* a modified GFP protein with an endoplasmic reticulum targeting sequence; *CaMV35Spro,* the cauliflower mosaic virus 35S promoter; *NosTer,* the terminator from nopaline synthasegene. The probe used in northern blot analysis of transgenic cells is the 816bp fragment of the *m-gfp5-ER* gene. The sequences of sense and antisense siRNA were indicated immediately below the *m-gfp5-ER* gene, and the position of siRNA was between nucleotides 183 and 203 of the *m-gfp5-ER* gene. **(B)** Northern blot analysis of total RNA from transgenic cell lines of rice (Ri), cotton (Co), Virginia pine (Vp), and eastern white pine (Wp). RNA (10µg) was extracted from transgenic cells of Ri, Co, Vp, and Wp at 15h after treatment with siRNAs, and were hybridized (at 65oC) with the 816-bp *m-gfp5-ER*probe corresponding to the *m-gfp5-ER* gene, which were labeled with DIG. The control panel is *gfp* transgenic cells 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). **(C)** Hybridization signals were quantified with a Storm 860PhosphorImager (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, Vp, and Wp 5 d after treatment. Values represent the means ± S.E.

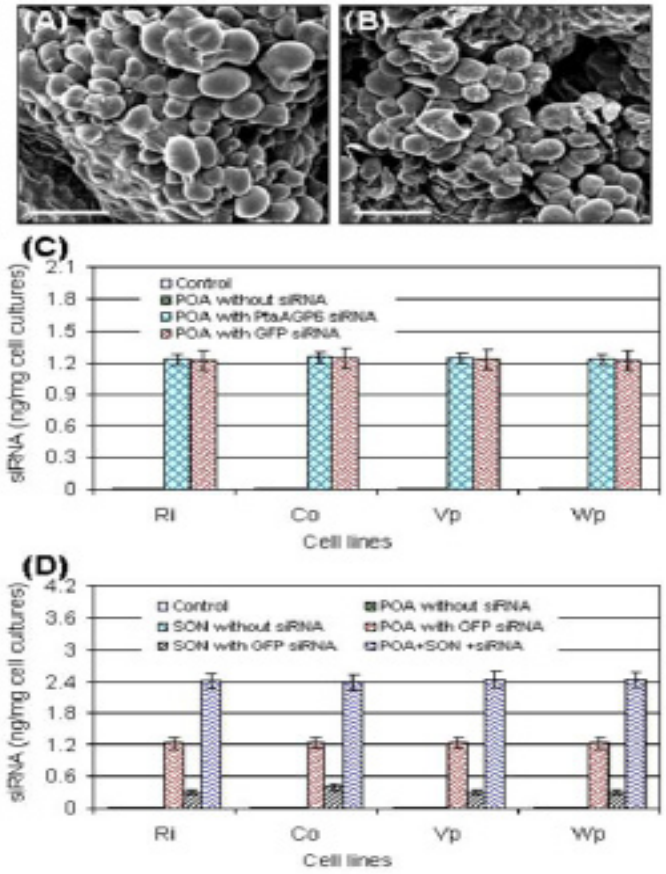

Figure 2: Scanning electronic microscopy and siRNA analysis. **(A)** Scanning electron microscopy of Virginia pine cells (Vp) that were not sonicated but treated by siRNA/POA complex. **(B)** Scanning electron microscopy of Virginia pine cells (Vp) that weresonicated and treated by siRNA/POA complex. **(C)** siRNA analysis of transgenic cell lines Ri, Co, Vp, and Wp 6 h after treatment with siRNA, which were not sonicated, **(D)** siRNA analysis of transgenic cell lines Ri, Co, Vp, and Wp 6 h after treatment with siRNA, which were treated by sonication, or POA, or sonication and POA, (A and B bars= 0.1 mm). Values represent the means ± S.E.

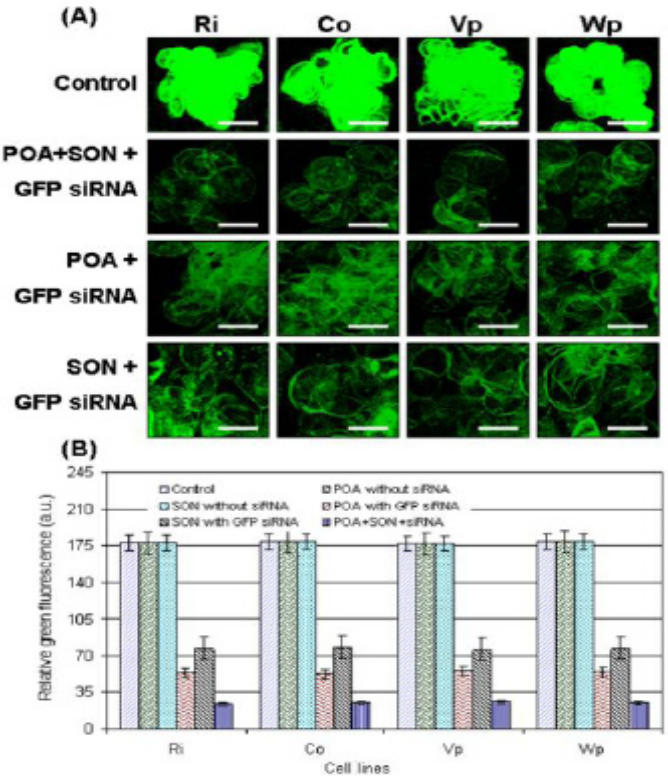

Figure 3: **(A)** Laser scanning microscopy of GFP expression silenced by siRNAs in transgenic cells 15h after treatment with siRNAs in transgenic cell lines Ri, Co, Vp, and Wp. Control, *gfp* transgenic cells without treatment with siRNA. GFP fluorescence was decreased more in all transgenic cells after sonicated and treated with siRNA/POA complex than sonicated or treated with siRNA/POA complex only (bars = 0.1mm). **(B)** Quantitative analysis of *gfp* expression in transgenic cells Ri, Co, Vp, and Wp 15 h after treated with siRNA. GFP fluorescence was expressed as fluorescence intensity (arbitrary unit). Fluorescence of *gfp* transgenic cells without treatment with siRNA was also presented as a control. Experiments were repeated three times, and each replicate consisted of 30-50 cells. Values represent the means ± S.E.

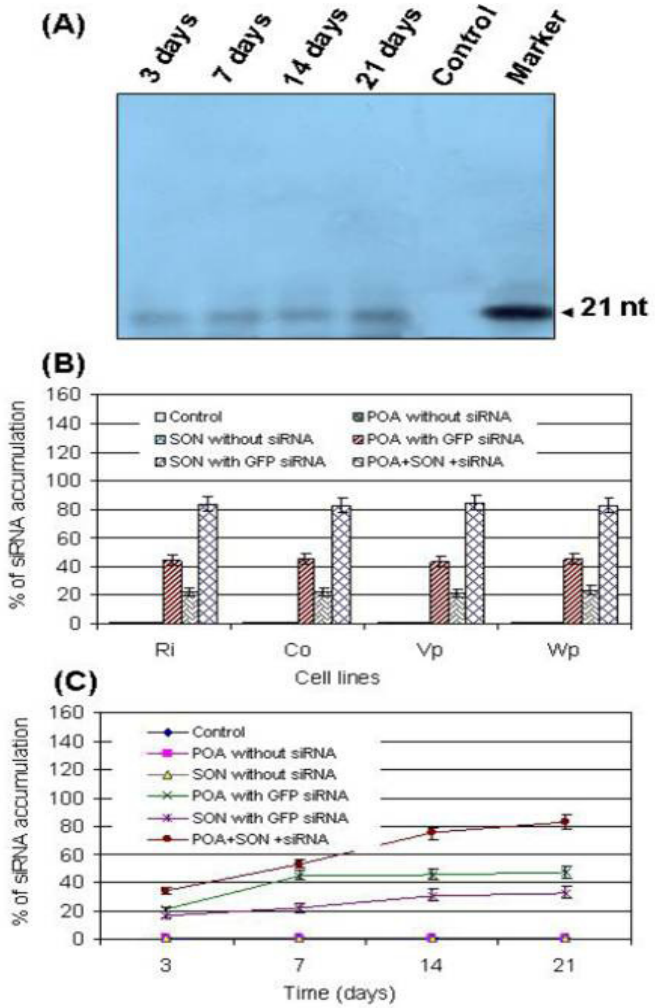

Figure 4: **(A)** Detection of small RNAs. Low molecular weight RNA fractions were isolated from transgenic cells of Virginia pine (PVf9), separated on polyacrylamide gels, blotted onto Hybond N+ membranes, and hybridized with 816bp *gfp*-coding sequences. The 2nt siRNA oligomers were used as size controls (size indicated in nucleotides). Eacnumbered lane contains the low molecular weight RNA fraction of transgenic cell treated with siRNA. No specific signal could be detected in transgenic cells without treatment of siRNAs with the probes. Lines 1-4, 3d, 7d, 14d, and 21d, after treatment with siRNA. Line 5, *gfp* transgenic cells without treatment with siRNA was presented as a control. Line 6 the 21-nucleotide small *gfp*-specific RNAs were as a marker. **(B)** siRNA analysis of transgenic cell lines Ri, Co, Vp, and Wp, which were treated by sonication, or POA, or sonication and POA, two weeks after treatment with siRNA. Values represent the means ± S.E. **(C)** siRNA analysis of transgenic cell lines Vp, which were treated by sonication, or POA, or sonication and POA, 3.d, 7d, 14d, and 21d after treatment with siRNA. Values represent the means ± S.E.

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