Transgenic plant-derived siRNAs can suppress propagation of influenza virus in mammalian cells

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Abstract As an example of the cost-effective large-scale generation of small-interfering RNA (siRNAs), we have created transgenic tobacco plants that produce siRNAs targeted to the mRNA of the non-structural protein NS1 from the influenza A virus subtype H1N1. We have investigated if these siRNAs, specifically targeted to the 5′-portion of the *NS1* transcripts (*5mNS1*), would suppress viral propagation in mammalian cells. Agroinfiltration of transgenic tobacco with an *Agrobacterium* strain harboring a *5mNS1*-expressing binary vector caused a reduction in *5mNS1* transcripts in the siRNA-accumulating transgenic plants. Further, H1N1 infection of siRNA-transfected mammalian cells resulted in significant suppression of viral replication. These results demonstrate that plant-derived siRNAs can inhibit viral propagation through RNA interference and could potentially be applied in control of viral-borne diseases.

Keywords: Transgenic plant; Small-interfering RNA; Gene encoding the non-structural protein NS1; Influenza virus; Mammalian cells; Anti-viral

1. Introduction

RNA interference (RNAi) is an ancient and evolutionarily conserved activity in eukaryotes. It results in RNA-mediated RNA degradation in a sequence-specific manner. Originally described in plants as a concerted inactivation of host genes and transgenes transcribing the same or similar sequences [1], it has been confirmed to occur in many different organisms. Examples include quelling in *Neurospora crassa* [2], and RNAi in *Caenorhabditis elegans* [3], *Drosophila* [4] and mammals [5]. In all these cases, RNAi is achieved through several closely coordinated steps: (1) an endonuclease Dicer with RNase III activity cleaves the dsRNA into 21–23 bp small interfering RNAs (siRNAs); (2) the siRNAs interact with a multicomponent nuclease to form an RNA-induced silencing complex (RISC); (3) the siRNA in the RISC directs the complex to the target RNA through sequence complementarity; (4) RNA polymerization begins from the siRNA to form dsRNA; and (5) the dsRNA is cleaved into siRNAs [6,7]. The resulting siRNAs would then initiate another round of RNA cleavage.

Studies using synthetic [8], in vitro transcribed [9,10] and in vivo transcribed [11,12] siRNAs, as well as viral-mediated siRNA delivery [13], have demonstrated that well-designed siRNAs can effectively suppress target gene expression. Hence, RNAi technology could eventually be applied in the therapeutics of human and animal viral diseases of which the molecular components, e.g., viral sequences, are known, and in the case of infectious diseases, of which the relevant pathogens have been identified. In plants, viral-resistance has already been achieved through a plant RNAi pathway termed post-trancriptional gene silencing (PTGS) [14].

Although some understanding on siRNA inhibition of viral propagation [8,11] has been achieved, the local folding of the target RNAs that reduces siRNA accessibility within a transcript [15] makes it necessary to test out many different siRNAs before optimal transcript degradation can be attained [8,16]. For example [8], 20 siRNA oligos were screened before identification of one that could satisfactorily suppress replication of the influenza virus in mammalian cells. Also, siRNA-mediated gene suppression in mammals requires the dsRNA to be smaller than 30 bp to ensure specificity [17], as long dsRNA can provoke non-specific degradation of RNA transcripts and a general shutdown of protein translation [18]. Therefore, it is impossible to transfect mammalian cells with long-dsRNA-producing constructs essential for making multiple siRNAs. The high cost in RNA oligo synthesis and the toxic effects of long dsRNA in mammalian cells could be ameliorated by cost-effective techniques in simultaneous large-quantity production of different siRNAs to achieve a satisfactory level for RNAi-mediated gene suppression.

Here, we demonstrate that tobacco (*Nicotiana tabacum*) can be engineered by *Agrobacterium*-mediated transformation to produce siRNAs targeting the mRNA for the non-structural NS1 protein of the influenza virus A/WSN/33, subtype H1N1. The transgenic plants could effectively accumulate siRNAs that specifically target gene encoding the non-structural protein NS1 (*NS1*) transcripts. Transfection of mammalian cells with plant-derived siRNAs followed by infection of the influenza virus revealed significant reduction in viral propagation.

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Abbreviations: NS1, gene encoding the non-structural protein NS1; MDCK, Madin–Darby canine kidney; siRNA, small-interfering RNA; RNAi, RNA interference; PTGS, post-transcriptional gene silencing; *EYFP*, gene encoding the enhanced yellow fluorescent protein

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Our data demonstrate that plants can be used as an economical and sustainable source for large-scale production of diversified siRNAs.

2. Materials and methods

2.1. Construction of hairpin RNA vector and generation of transgenic tobacco plants

A 0.4-kb fragment representing the 5′-portion of the *NS1* mRNA (*5mNS1*) from the influenza virus strain A/WSN/33 subtype H1N1 (Fig. 1A and B) was amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) using forward primer 5′-ggg*cggccgc***ggatcc**atggacccaaacactgtg-3′ with *Not*I (in italics) and *Bam*HI (in bold) sites incorporated at its 5′-end, and reverse primer 5′-ca*actagt*atttcgctttcagtatga-3′ with an added *Spe*I site (in italics). The underlined nucleotides represent *NS1* sequences. The PCR product was initially cloned in pGEM-T Easy vector (Promega) for verification of DNA sequence. Subsequently, the pGEM-T Easy derivative was digested with either *Bam*HI and *Spe*I or *Not*I and *Spe*I. The 0.4-kb *Bam*HI–*Spe*I *5mNS1* fragment was cloned into corresponding sites in a pBluescript SKII(−) derivative that contains the *Arabidopsis TGA1* intron [19] inserted at its *Spe*I–*Xba*I site. Next, the 0.4-kb *Not*I–*Spe*I *5mNS1* fragment from the pGEM-T Easy derivative was cloned in the *Not*I–*Xba*I site of the pBluescript SK(II)(−) derivative containing the DNA fusion of "sense *5mNS1-TGA1* intron", to generate a dsRNA cassette "sense *5mNS1-TGA1* intron–antisense *5mNS1*". This cassette was then released by *Not*I and *Bam*HI digestion, and, with the help of a *Not*I/*Xba*I adaptor (upper strand, 5′-GGCCGAGTTGTTA-3′; lower strand, 5′-CTAGTAACAACTC-3′), was cloned in the *Bam*HI–*Xba*I site between the *CaMV* 35S promoter and the *nos* terminator, in another pBluescript SKII(−) derivative. The resulting vector therefore contains a cassette of "35S-s *5mNS1–TGA1* intron–as *5mNS1 nos*" (Fig. 1C). This cassette was further digested with *Not*I and *Kpn*I, and was cloned into corresponding sites within the T-DNA in a pBI101 backbone plasmid derivative (Clontech, Palo Alto, USA). The binary vector was then mobilized into *Agrobacterium tumefaciens* strain GV3101/MP90 for transformation of tobacco cultivar Samsun NN by the leaf-disk procedure [20].

2.2. siRNA detection

Total RNA samples were extracted from tobacco leaves using TRIzol (Invitrogen). Twenty micrograms of total RNA was separated on a 15% polyacrylamide gel containing 7 M urea and was electroblotted onto a nitrocellulose membrane (GeneScreen Plus®, PerkinElmer Life Sciences, Inc.). The blot was then hybridized overnight at 42 °C to [^{32}P]UTP-labeled *5mNS1* riboprobes generated using the Riboprobe® in vitro Transcription Systems (Promega), in a solution of 50% (v/v) formamide, 250 mM NaCl, 7% SDS and 125 mM phosphate buffer, pH 7.0. After hybridization, the blot was washed twice with 2× SSC plus 0.5% SDS and was then analyzed using a phospho-imager. The volumes of the synthetic siRNA and of the siRNA from transgenic plants were measured using an ImageQuant software (Molecular Dynamics), and the amount of siRNA in the plant RNA sample was calculated based on its volume relative to that of synthetic, known amount of RNA oligos.

2.3. Transient expression assay by agroinfiltration

Agrobacterium cells containing the *5mNS1*-expressing binary vector and those containing an *EYFP-T2m* (*EYFP*, gene encoding the enhanced yellow fluorescent protein) expressing binary vector [21] were inoculated in an induction solution containing 1 g/l NH_4Cl, 0.3 g/l $MgSO_4.7H_2O$, 0.15 g/l KCl, 0.01 g/l $CaCl_2$, 0.0025 g/l $FeSO_4 \cdot 7H_2O$, 2 mM phosphate, 1% glucose, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 5.5), 100 μM acetosyringone, 50 μg/ml kanamycin and 50 μg/ml gentamycin. The *EYFP-T2m* contains *EYFP* fused in-frame to a mutant version of the *Arabidopsis TGA2* gene (*T2m*) and is used as an expression reference after agroinfiltration. Following overnight culture at 28 °C, the cells were collected by centrifugation at 3000 × *g* for 15 min, and then resuspended in an infiltration solution containing 10 mM MES (pH 5.5), 10 mM $MgSO_4$ and 100 μM acetosyringone. The resuspended *Agrobacterium* cells were adjusted to an OD_{600} of 0.8 with the same solution before infiltration of tobacco leaves using a 1 ml syringe. After two days, total RNA was extracted from the infiltrated leaf areas for Northern blot analysis.

2.4. Northern blot analysis

Five micrograms of total RNA, extracted from the agroinfiltrated and non-infiltrated leaf areas, were separated on a 1.2% agarose gel, blotted with 20× SSC onto a nitrocellulose membrane, and hybridized to [^{32}P]dCTP-labeled DNA probes generated from *5mNS1* and *EYFP* DNA fragments using a Rediprime™ II Random Prime Labelling System (Amersham, UK). Hybridization was performed at 65 °C overnight in a buffer containing 250 mM NaCl, 7% SDS and 125 mM phosphate, pH 7.0. After hybridization, the blot was washed twice at room temperature in 2× SSC plus 0.5% SDS, then at 65 °C for 15 min in 0.2× SSC plus 0.1% SDS. The blot was analyzed using a phospho-imager.

2.5. Transfection of mammalian cells followed by infection with influenza virus

Confluent Madin–Darby canine kidney (MDCK) cells grown in a T-175 flask were washed twice with phosphate-buffered saline (PBS) and trypsinized for 10 min in 10 ml trypsin solution at 37 °C. After ter-

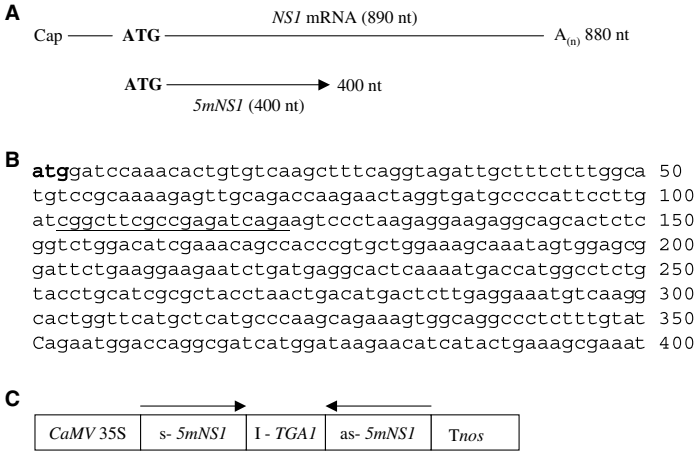

Fig. 1. The *5mNS1* sequence and the hairpin RNA construct used in producing *5mNS1* siRNAs in tobacco. (A) Schematic representation of *NS1* mRNA. The cap and poly(A) tail structures are shown, and location of the 0.4-kb *5mNS1* fragment beginning from the first codon (**atg**) is indicated. (B) cDNA sequence of the *5mNS*, with the sequence of the synthetic siRNA NS-128 used by Ge et al. [8] underlined. (C) Diagram showing RNAi cassette in a binary vector. The sense (S) and antisense (AS) *5mNS1* fragments are separated by the *Arabidopsis TGA1* intron (I-*TGA1*), and are under the control of the *CaMV* 35S promoter.

mination of trypsinization with 20 ml PBS, the cells were collected by centrifugation for 5 min at 15 000 rpm, and were washed twice in 30 ml cold PBS, followed by one wash in 30 ml cold RPMI1640 medium (Gibco), before resuspension in cold RPMI1640 to a density of 1×10^7 cells/ml. Subsequently, 500 μl of resuspended cells was transferred into a 0.4 cm pre-chilled cuvette, and was mixed with 10 μl water, 10 μl water with 42 ng NS-128, 10 μl wild-type RNA sample or 10 μl RNA sample containing 42 ng siRNAs from transgenic plant. Equal amounts of total RNA from wild-type or transgenic plants were used. The cuvette was kept on ice for 10 min, before electroporation at 0.4 kV and 960 μF using a gene pulser system (Bio-Rad). Cells were then transferred into 5.6 ml of pre-warmed MDCK medium (MEM, 10% cow serum, 1% penicillin and 1% streptomycin). Three milliliters was transferred into a 6-well plate and incubated at 37 °C for 24 h before infection with the influenza virus.

2.6. Virus infection and hemagglutination (HA) titer test

Twenty-four hours after transfection, cells in each well were washed twice with PBS, and 300 μl diluted influenza virus strain A/WSN/33 (MOI = 0.001 in PBS) was added into the well. After shaking the mixture for 1 h, the viruses in the supernatant were discarded, and 2 ml infection medium [0.5 μg/ml TPCK-trypsin (Sigma), 0.5% FCS (Gibco), and 1% PS with MEM (Gibco)] was added into the well. The cells were then incubated at 37 °C. Supernatants were collected at different post-infection time points for the HA titer test as described [8].

3. Results and discussion

Influenza A viruses are medically important viral pathogens that cause significant mortality and morbidity throughout the world. Their easy transmission, antigenic shift and drift have made current methodology of vaccination and therapy limited in efficacy [22]. Inhibitors of the anti-M2 ion channel and neuraminidase are common drugs for influenza, but both have their drawbacks. The anti-M2 ion channel inhibitors (e.g., amantidine) induce viruses to develop drug-resistant mutations, while the neuraminidase inhibitors (e.g., Tamiflu), though very potent, are effective only at early disease onset. To investigate if plant-derived siRNAs against the influenza virus could inhibit viral replication, we selected a 0.4-kb fragment representing the 5′-portion of the *NS1* gene in strain A/WSN/33, subtype H1N1. The NS virion RNA (vRNA) consists of about 890 nucleotides and encodes two non-structural proteins, NS1 and NS2. The sequence of this vRNA is highly conserved among different subtypes of influenza viruses [23]. The NS1 protein has not only been proposed to regulate viral replication cycle, splicing and translation of mRNAs [24], but also been shown to have inhibitory effect on cellular mRNA maturation and cellular anti-viral response [25]. Thus, the NS1 gene plays an important role in virus replication and virus–host interactions. The chosen 0.4-kb fragment was amplified by PCR, and then sequentially cloned in sense and antisense orientations, on either side of the *Arabidopsis TGA1* intron. The resulting cassette of "sense–intron–antisense" was expressed from the *CaMV* 35S promoter in a binary vector (Fig. 1C). Hence, transgenic plants obtained in *Agrobacterium*-mediated plant transformation from this binary vector should produce hairpin dsRNA, which would subsequently be processed into siRNAs by the PTGS machinery.

Reports have shown that "sense–antisense" cassettes can be transcribed to produce siRNAs after transfection of host cells [11,26,27]. To investigate if the construct generated in this study (Fig. 1C) could produce siRNA in transgenic tobacco, RNAs from leaves of primary transformants was separated on a gel of 15% polyacrylamide and 7 M urea, blotted onto nitrocellulose membrane and hybridized to [^{32}P]UTP-labeled *5mNS1* riboprobes. Of 21 independent transformants screened, 13 showed obvious siRNA production. The levels of siRNA accumulation in different lines varied, some produced obvious signals after an overnight exposure using a phosphoimager, while others barely yielded visible signals (data not shown). The siRNA signals in selected transgenic lines are shown in Fig. 2. Transgenic lines 1 and 2 had apparent accumulation of *5mNS1* siRNAs, while lines 8 and 9 produced much lower levels of the same siRNAs. In line 10, the siRNAs were barely detectable.

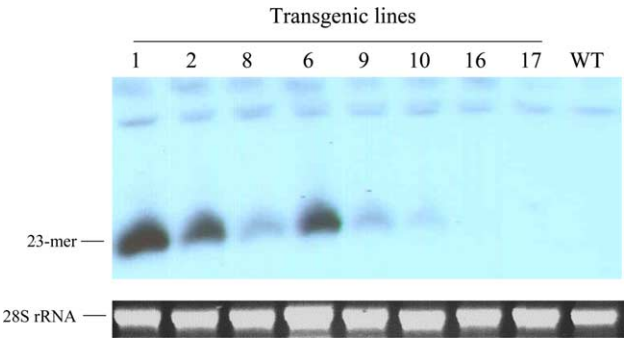

Fig. 2. Accumulation of the *5mNS1* siRNA in selected primary transformants and in wild-type tobacco (WT). (A) Twenty micrograms of total RNA from leaves of transgenic tobacco was separated on a 15% polyacrylamide gel containing 7 M urea, blotted and hybridized to [^{32}P]UTP-labeled *5mNS1* riboprobes. (B) Normalization of RNA loading was based on the separation of 6 μg of total RNA on a 1.2% agarose gel.

A variation in siRNA levels may be due to several reasons. First, T-DNA location in the genome could affect expression. In *Agrobacterium*-mediated plant transformation, T-DNA is transferred from the bacterium to the eukaryotic host cell and further integrated into the host genome [28]. If the transgene were inserted in the genome where active transcription occurs, the transgene would be active. Otherwise, it would be less active or even silent. Second, the copy number of the transgene may be a contributing factor in expression levels, although in some cases, transgene activity may not be directly proportional to its copy number due to co-suppression. Third, methylation of transgene may occur, especially at or near promoter if it is considered foreign. As a safeguard, the host generally has a mechanism to methylate and inactivate the transgene. This has been reported with foreign DNA expressing dsRNA in PTGS [29,30] and is supported by a requirement of DNA methylase in initiating RNA-dependent DNA methylation [31].

As revealed by an increasing number of reports, siRNA is the hallmark in triggering RNAi. Therefore, the accumulated *5mNS1* siRNAs in the transgenic plants should initiate degradation of *NS1* transcripts or endogenous tobacco transcripts with sequences complementary to *5mNS1*. A BLAST analysis was performed with *5mNS1* as query sequence for such complementation in transcripts of tobacco or species evolutionarily close to tobacco, but no match was identified. Northern blot analysis of tobacco total RNA with the *5mNS1* probe also did not yield any obvious bands. Therefore, *5mNS1* does not seem to share homology to any tobacco transcripts and would not cause unintended degradation of RNA transcribed from endogenous genes. This is consistent with the fact that no abnormal phenotypes were observed in all the transgenic lines

(data not shown). To test if the plant-derived *5mNS1* siRNAs were functional in degrading *NS1* transcripts specifically, *5mNS1*- and *EYFP-T2m*-expressing binary vectors were introduced into *Agrobacterium* cells which were used to co-infiltrate leaves of wild-type tobacco and those of transgenic tobacco lines expressing *5mNS1* siRNAs. As shown in Fig. 3B, all the three transgenic lines 1, 9 and 19, representing high, middle and low accumulation of *5mNS1* siRNA, respectively, had reduced *5mNS1* RNA levels, indicating that plant-derived *5mNS1* siRNAs indeed triggered PTGS of *NS1* in vivo. A negative correlation was observed between the levels of *5mNS1* siRNAs and *5mNS1* transcripts in infiltrated tobacco leaves. To obtain a percentage of the *5mNS1* transcript level in the transgenic lines relative to that of wild-type, volumes of each *5mNS1* band and of the reference *EYFP* band were determined using the ImageQuant software, and percentage was calculated using the formula described in Fig. 3 legend. In transgenic tobacco line 1, which had the highest level of *5mNS1* siRNA accumulation, the percentage was only 0.4%, demonstrating high efficiency of this line in *5mNS1*-specific RNA degradation (Fig. 3C).

In RNAi studies, synthetic or in vitro expressed siRNAs have been used in transfection of target cells [26], and injection of worms [32] and animals [33], for evaluation of siRNA efficacy. To test if the *5mNS1* siRNAs produced in transgenic tobacco could be potentially used in suppressing viral propa-

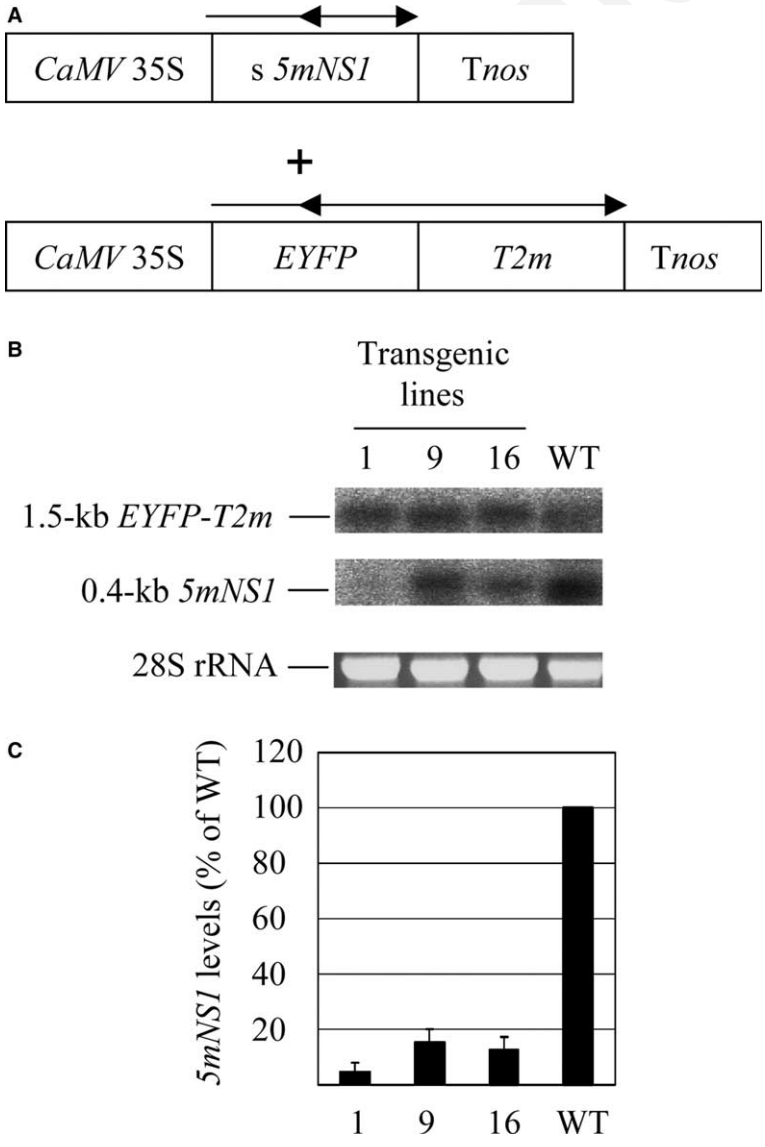

Fig. 3. Suppression of *5mNS1* transcript accumulation in siRNA-expressing lines. Wild-type tobacco and transgenic plants expressing different levels of *5mNS1* siRNAs were co-infiltrated with two binary vectors separately expressing *5mNS1* and a fusion fragment of *EYFP T2m*. After two days, leaf samples were collected for RNA analysis by Northern blot analysis. (A) Part of the T-DNA in the two binary vectors. (B) Northern blot analysis showing levels of *EYFP-T2m* and *5mNS1* transcripts in the different infiltrated samples. The 28S rRNA was stained with ethidium bromide. The 5mNS1 levels are lowered in transgenic plants when compared to levels in WT. (C) The *5mNS1* transcript level, as a percentage of the wild-type, was calculated with data from three separate infiltrations. Calculation was performed according to the formula of:

$$5mNS1 \text{ level (\% of WT)} = \frac{(100) \times (5\ mNS1 \text{ volume of transgenic line}) \times (EYFP \text{ volume of wild-type})}{(5mNS1 \text{ volume of wild type}) \times (EYFP \text{ volume of transgenic line})}.$$

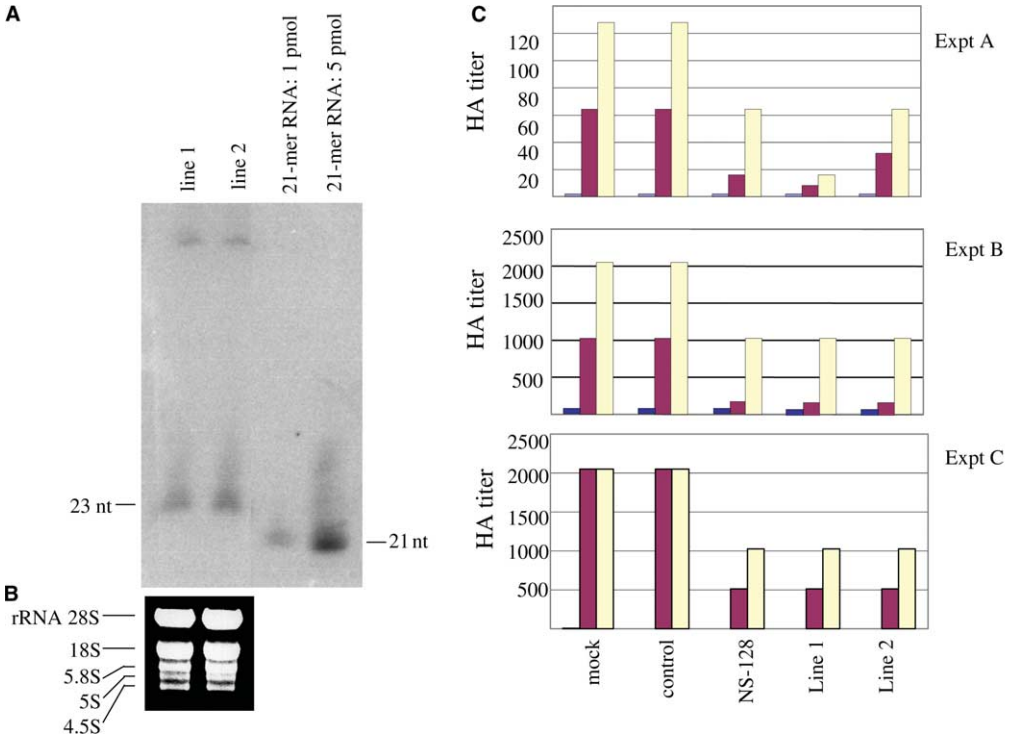

Fig. 4. Plant-derived *5mNS1* siRNAs can suppress replication of the influenza virus A/WSN/33 in mammalian cells. (A) Total RNA (10 μg) from primary transformants 1 and 2 was separated on a 15% polyacrylamide gel, blotted onto a Nylon membrane and probed with [^{32}P]UTP-labeled *5mNS1* RNA probes. Quantity of siRNAs in the RNA samples was calculated based on its relative volume to that of known amount of synthetic siRNA oligo. These RNA preparations were then used for transfection of MDCK cells. (B) Normalization of RNA loading was based on the separation of 6 μg of total RNA on a 1.2% agarose gel. (C) Suppression of viral replication as revealed in three independent HA titer assays. MDCK cells were first transfected with water (mock), a siRNA oligo NS-128 used by Ge et al. [8], and RNA from wild-type tobacco (control) or from two transgenic lines (line 1 and line 2) expressing siRNAs, and were then infected by influenza virus strain A/WSN/33 24 h post-transfection. HA titer was determined at 24 (blue boxes), 36 (red boxes) and 48 h (yellow boxes) post-infection.

gation in mammalian cells infected with the influenza virus, *5mNS1* siRNAs were harvested from the leaves of transgenic plants for transfection of MDCK cells. The amount of siRNA in total RNA was quantified by siRNA analysis (Fig. 4A). As a positive control, we used siRNA oligo NS-128 (5′-CGGCUUCGCCGAGAUCAGAdAdT-3′), since it has been proven best of three *NS1*-targeting siRNA oligos [8]. Cells transfected with RNA from non-transformed plants were the negative control and cells transfected with water constituted the mock transfection. Twelve hours after transfection, cells were infected by the influenza virus strain A/WSN/33 (MOI = 0.001). The HA titer, which is an indicator of viral replication, was determined at 12, 24 and 36 h post-infection. The mock-transfected and the negative control cells showed similar HA titer, indicating that RNA from wild-type tobacco plants did not suppress viral replication. Though the HA titer values varied in three separate sets of transfection and infection studies, a phenomenon unavoidably associated with conditions of the cells, e.g., passage history, both plant-derived and synthetic siRNAs significantly reduced H1N1 viral replication. The anti-viral effect of siRNA was most prominent at 36 h post-infection (Fig. 4B). In one set of experiments, plant-derived siRNA proved superior to the NS-128 oligo (Fig. 4B, experiment A).

These results strongly support our hypothesis that *5mNS1* siRNA from transgenic plants can effectively suppress replication of the influenza virus in mammalian cells. In addition, plant siRNAs showed similar suppression ability as the synthetic siRNA NS-128, demonstrating that plant-derived siRNAs confer the same efficacy. Given the fact that transgenic plants can generate siRNAs targeting different areas of the *5mNS1* transcript, and that *NS1* sequences are highly conserved among influenza viruses [23], *5mNS1* siRNAs from transgenic plants should suppress the replication of a broad range of influenza viral subtypes with sequences homologous to the *5mNS1*.

While our results clearly indicate anti-viral effects of plant-derived *5mNS1* siRNAs, this study is primarily focusing on developing a strategy for economical and sustainable production of siRNAs. Besides using transgenic technology described in this study, a pool of siRNAs can also be generated with Dicer-dependent kits. When compared with the transgenic approach, the latter method is much more expensive, since it involves expensive reagents (i.e., dNTP, Dicer, and RNA polymerase), complicated steps (i.e., in vitro transcription, in vitro cleavage of dsRNA, and clean-up) and experienced researcher. The high cost not only limits production scale, but also requires repetition of the production process if the siRNAs are to be used over and over again. Therefore, our proof-of-concept study demonstrates that transgenic plants are superior to the commercial kits for siRNA production and the time taken for generating them would be well compensated.

Though the *NS1*-targeting siRNAs possess anti-viral effects, those against the NP genes would be more potent in suppressing viral replication, as revealed by studies using mammalian cells [8] and animals [34]. In both studies, one of the NP-targeting siRNAs, Np-1496, significantly reduced the virus titers. These observations indicate that mRNA of the NP gene might be a better target of siRNA, if positional effects on siRNA accessibility could be faithfully addressed. In our future study of using transgenic plant-derived siRNAs for viral suppression, generating NP-targeting siRNAs would be a more practical practice.

In conclusion, *5mNS1* siRNAs capable of activating RNAi in mammalian cells against *NS1* were produced in transgenic tobacco plants. The efficacy of the plant-derived siRNAs was tested in vivo by agroinfiltration of the *5mNS1*-expressing construct in leaves of transgenic tobacco and in vitro by application of these siRNAs in mammalian cells to inhibit influenza viral replication. This cost-effective technique in utilizing transgenic plants for large-scale siRNA production could have advantages over current methods involving the use of synthetic RNA oligos, the expression of short hairpin RNA in *Escherichia coli* [35,36] and the transfection of mammalian cells with short dsRNA. In addition, plant cells can apparently tolerate expression of long dsRNAs, enabling the length of the target gene fragment to be easily manipulated for optimal suppression. Moreover, fragments producing siRNAs targeting multiple sites of the viral genome can be fused together so that one transgenic plant can produce siRNAs for simultaneous silencing of multiple genes. This could provide a more robust and sustained viral protection minimizing the likelihood of the virus developing resistance to the siRNA through mutation of the target sequence.

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