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A Viral Protein Suppresses siRNA-directed Interference in Tobacco Mosaic Virus Infection

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Abstract Plant viruses encode suppressors of post-transcriptional gene silencing (PTGS), an adaptive defense response that limits virus replication and its spread in plants. The helper component proteinase (HC-Pro) of the potato virus A (PVA, genus *Potyvirus*) suppresses PTGS of silenced transgenes. Here, the effect of HC-Pro on siRNA-directed interference in the tobacco mosaic virus (TMV) was examined by using a transient *Agrobacterium tumefaciens*-based delivery system in intact tissues. It was shown that the interference effect was completely blocked by co-infiltration with HC-Pro plus siRNA constructs in both systemic and hypersensitive hosts. In the system host, all plants agro-infiltrated with HC-Pro plus siRNA constructs displayed the same symptoms as the negative control. Meanwhile, TMV RNA accumulation was found to be abundant in the upper leaves using reverse transcriptase-PCR (RT-PCR) and Northern blot assays. On the contrary, plants agro-infiltrated with the siRNA construct alone were free of symptoms. Therefore, our study suggests that the transient expression of HC-Pro inhibited the siRNA-directed host defenses against TMV infection.

Key words siRNA; HC-Pro; tobacco mosaic virus; suppressor

RNA interference (RNAi) is a double-stranded RNA (dsRNA)-induced gene-silencing phenomenon that is conservative among various organisms, including animals and plants [1]. *In vivo*, RNAi is initiated by an endonulease, Dicer, which cleaves long double-stranded RNA into 21- to 25-nt small interference RNA (siRNA) [2–5]. Recently, it has been shown that siRNA of 21 nt in size, an intermediate of the RNA-interference pathway, is effective in the inhibition of viral infection and modulation of viral replication in cultured mammalian [6–8] and plant cells [9]. This discovery has been proposed to be amenable for antiviral purposes. The antiviral activity of siRNA has also been demonstrated in several recent studies to be directed against the human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV) [10–12].

In plants, RNAi is referred to as post-transcriptional gene silencing (PTGS) and thought to be a natural line of defense against viral infection [13]. As a response to this defense mechanism, many viruses encode gene-silencing suppressor proteins acting at different points in the PTGS pathway [14]. In recent years, it has been shown that the helper component proteinase (HC-Pro) of potyviruses is a suppressor of the PTGS pathway [15,16], which interferes with a maintenance stage in the silenced state. Moreover, RNA silencing triggered by a double-stranded *GFP* transcript transiently expressed by agro-infiltation was partially inhibited by the HC-Pro of the tobacco etch virus (TEV). The plants expressing the transgene HC-Pro of the potato virus A (PVA), a member of genus *Potyvirus*, were initially

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susceptible to infection of homologous viruses, but later exhibited strong silencing of both the transgene and the homologous viruses, which was revealed as a recovery from infection [17]. In addition, the 19 kDa protein (P19) of tombusviruses can bind PTGS-generated dsRNAs (21–25 nt) and synthetic dsRNAs (21 nt) or blunt-ended dsRNAs (21 nt) [18].

The tobacco mosaic virus (TMV) has infected a wide variety of economically important crops worldwide. This virus has a single-stranded RNA genome. Among the different products encoded by the virus, the 126 kDa and 183 kDa proteins, which are the replication-associated proteins, are indispensable for viral RNA replication. Therefore, we envisaged the use of siRNA to target the open reading frame (ORF), which could be an effective way to counter TMV. We have previously shown that the siRNA of the 126 kDa protein derived from the TMV genome can interfere with virus infection in a sequence-specific manner using the *Agrobacterium tumefaciens* infiltration system.

In this study, we performed the co-infiltration assay to determine whether the transient expression of HC-Pro *o*f PVA can block the interference in TMV infection in tobacco directed by the siRNA of its 126 kDa protein.

Materials and Methods

Plasmids and their construction

The basic vector for all constructs, pBI121 (a generous gift from Dr. Francisco TENLLADO; Departamento de Biologia de Plantas, Centro de Investigaciones Biologicas, CSIC, Madrid, Spain), contains an enhanced 35S promoter from the cauliflower mosaic virus, the β-glucuronidase (GUS) gene and the 35S terminator.

pBI121 vector was used to generate short, unimolecular RNA transcripts which serve as siRNAs. To design target-specific siRNA duplex against 126 kDa protein, we selected sequence of the type AA(N21) (N, any nucleotide) from the coding sequence of 126 kDa mRNA. Positions 1519 to 1538 relative to the transcription start site were suitable for the design of a specific siRNA-directed against TMV 126 kDa protein. The selected siRNA sequence was also submitted to BLAST search to ensure that it was specific to the target mRNA. Oligonucleotides contained both the 19-nt sense and 19-nt antisense strands separated with a short spacer from the reverse complement of the same sequence and five thymidines as termination signal. The primers used were: sense 5′-GATCCAAGCTTCG*ACTTATCAGAGTGGCAGGC***TT**CAAGAGA-G*CCTGCCACTCTGATAAGTT*TTTTGAGCT-3′ and antisense 5′-CAAAAA*ACTTATCAGAGTGGCAGGC*TCT-CTTG**AA***GCCTGCCACTCTGATAAGT*CGAAGCTTG-3′. The complementary target sequences of 126 kDa protein and thymidines are in italic and in bold, respectively. These primers which, after annealing, generate double-stranded DNA fragments, were cloned into *Bam*HI/*Sst*I-digested pBI121 vector. The resulting transcript is predicted to fold back on itself to form a 19-bp stem-loop structure and the stem-loop precursor transcript is quickly cleaved in the cell to produce a functional siRNA.

The HC-Pro coding sequence was obtained by polymerase chain reaction (PCR) from the pKOH122 plasmid, which was a generous gift from Dr. Eugene I. SAVENKOV (Department of Plant Biology, Genetics Centre, Sweden). The primer sequence for HC-Pro were 5′-GCTGGA-TCCGCGGCCTTCAACA-3′ (forward primer) and 5′-GCTGAGCTCGGCCGGCCTATCCAA-3′ (reverse primer). The PCR fragment was digested with *Bam*HI and *Sst*I, and then inserted into the pBI121 vector, generating pBI121/HC-Pro.

Infiltration of *Agrobacterium tumefaciens*

A. tumefaciens infiltration assays were performed as described previously [19]. pBI121/siRNA and pBI121/HC-Pro constructs were introduced into the *A. tumefaciens* strain EHA105, which is provided by Prof. Qun LI (Institute of Plant Physiology and Ecology, SIBS, CAS, China), by direct transformation. The resulting *A. tumefaciens* was grown overnight at 28 °C in a tube containing 5 ml of Luria-Bertani medium supplemented with 50 μg/ml kanamycin. The cells were precipitated and resuspended to a final concentration of A_{600}=0.8 in a solution containing 10 mM $MgCl_2$, 10 mM 2-morpholinoethanesulfonic acid (MES), pH 5.6, and 150 μM acetosyringone. The cell solutions were incubated at 28 °C for 2 to 3 h before infiltration. By using a 5 ml syringe, the *A. tumefaciens* cell cultures carrying the pBI121/siRNA and pBI121/HC-Pro constructs were co-injected into the leaves of healthy *Nicotiana tabacum* tobacco plants (The Institute of Phytopathology, Northwest Sci-Tech University of Agriculture and Forestry, Shaanxi, China), through an incision made by a pinhead. Two leaves of each plant were infiltrated in the entirety and the whole plant was covered with a transparent plastic bag for 2 days.

Virus inoculation

TMV particles were isolated from systemically infected *N. tabacum* tobacco plants and purified by polyethlene

glycol (PEG) precipitation. Standard inoculation [19] was performed using 10 μg/ml purified viruses as the inoculum. The inoculation was performed on two fully expanded leaves of the tobacco plant that was infiltrated by *A. tumefaciens* by rubbing the leaf surface with the inoculum, using carborundum as an abrasive. The inoculated plants were kept in a growth chamber at 25 °C with 16 h of light and 8 h of darkness.

RT-PCR analysis of viral RNA in tobacco

The total RNA was extracted from upper leaves of tobacco (0.1 g) using the Trizol reagent (Invitrogen, California, USA). Reverse transcriptase-PCR (RT-PCR) was performed using the *Thermococcus kodakaraensis* (KOD)-plus DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The primers used to amplify the sequence corresponding to nucleotide 118 to 638 of TMV 126 kDa ORF were 5′-GCTCGTGAC-CGCAGGC-3′ (forward primer) and 5′-CGAACTCAT-CGGCTGGTAT-3′ (reverse primer).

Northern blot analysis of viral RNA in tobacco

The RNA samples (approximately 20 μg) were separated on 1% agarose formaldehyde gel, using a buffer consisting of 20 mM 3-(*N*-morpholino)-propane sulfonic acid (MOPS), 5 mM NaAC, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 7.0, transferred to Hybond-N membranes (Amersham), and subjected to UV cross-linking. The RNA blots were pre-hybridized in Church buffer at 65 °C for 1 h. Radiolabeled probes for the TMV 126 kDa ORF coding sequence were made by a random priming reaction in the presence of α-^{32}P-dATP, and were used to blot the RNA. Hybridization was done overnight in a rotating incubator at 65 °C, and this was followed by four washes (20 min each) in 2×standard saline citrate (SSC) buffer and 0.2% (*W*/*V*) sodium dodecyl sulfate (SDS) at 65 °C, 65 °C, 60 °C and 50 °C, respectively. The blots were scanned using a PhosphorImager (Storm860, Amersham Bioscience Company, USA).

Results

Transient expression of HC-Pro of PVA suppresses siRNA-directed interference in TMV infection

In order to determine if the transient expression of HC-Pro of PVA in plants was sufficient to inhibit siRNA-directed interference in TMV infection, equivalent volumes of the *A. tumefaciens* culture containing pBI121/HC-Pro and the *A. tumefaciens* culture containing pBI121/siRNA were mixed and infiltrated into the leaves of *N. tabacum* plants. To serve as controls, *N. tabacum* plants were infiltrated with an *A. tumefaciens* mixture containing pBI121/siRNA plus the pBI121 vector or an *A. tumefaciens* culture carrying an empty vector. At 4 days post-infiltration, TMV particles were directly inoculated onto the entire infiltrated leaf. In ten dependent experiments, all plants co-infiltrated with pBI121/HC-Pro and pBI121/siRNA were infected by TMV, and displayed typical symptoms of TMV infection in the upper leaves at 7 days post-inoculation (dpi), whereas 36 of 40 (90%) plants that were co-infiltrated with the pBI121/siRNA construct and the pBI121 empty vector showed no symptoms. Mosaic symptoms also appeared on the plants agro-infiltrated with the pBI121/HC-Pro construct and pBI121 [**Fig. 1(A)**].

The HC-Pro construct was also tested for its ability to suppress the interference effect in TMV infection directed by the siRNA targeting TMV 126 kDa protein in *Nicotiana glutinosa*, a hypersensitive host. The interference effect was completely blocked by co-infiltration with HC-Pro plus siRNA constructs, whereas no local lesion was found in the opposite half of the leaf co-inoculated with pBI121 and the siRNA construct as displayed [**Fig. 1(B)**]. The above results showed that the interference in TMV infection triggered by siRNA was suppressed by the transient expression of HC-Pro of PVA.

To detect the accumulation of TMV RNA in the upper leaves of *N. tabacum* plants, we further performed RT-PCR and Northern blot assays to confirm the suppression effect of HC-Pro. Using RT-PCR, the TMV was specifically separated in the leaves infiltrated with pBI121/siRNA plus pBI121/HC-Pro constructs, pBI121/HC-Pro construct plus pBI121 and pBI121 at 15 days post-inoculation. However, plants infiltrated with *A. tumefaciens* carrying the siRNA construct alone and the siRNA construct plus pBI121 could block TMV infection [**Fig. 2(A)**]. In the plants infiltrated by pBI121/siRNA or pBI121/siRNA plus pBI121, we detected nothing in the leaves. On the contrary, plants infiltrated by pBI121/siRNA plus pBI121/HC-Pro constructs, pBI121/HC-Pro plus pBI121 and pBI121 alone could not defend themselves against TMV infection [**Fig. 2(A)**, lanes 1, 4 and 5]. The results of the Northern blot analysis of total RNA extracted from inoculated leaves were consistent with that of the RT-PCR [**Fig. 2(B)**].

To investigate the degree of suppression of siRNA-directed interference in TMV infection by HC-Pro in the leaves of *N. glutinosa*, a hypersensitive host, leaf halves were mock inoculated or were inoculated with plant sap derived from the leaves of *N. tabacum* plants infiltrated

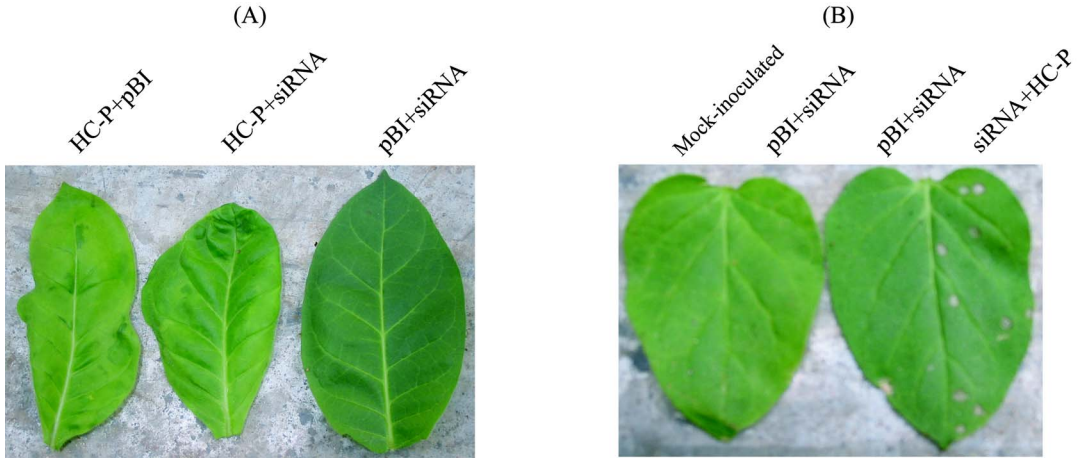

Fig. 1 Suppression of siRNA interference in tobacco mosaic virus (TMV) infection by the helper component proteinase (HC-Pro) gene of PVA

(A) The *Nicotiana tabacum* leaves were initially infiltrated with *Agrobacterium tumefaciens* cultures containing pBI121/HC-Pro plus pBI121 (HC-P+pBI), pBI121/HC-Pro plus pBI121/siRNA constructs (HC-P+siRNA) and pBI121 plus pBI121/siRNA construct (pBI+siRNA) as indicated. After 4 days, these leaves were inoculated with the tobacco mosaic virus (TMV). The upper infiltrated leaves were photographed at 14 days post-inoculation. (B) Response of the *Nicotiana glutinosa* to TMV infection. Half of the leaf was mock inoculated or infiltrated with pBI121+pBI121/siRNA, and the opposite half was infiltrated with pBI121+pBI121/siRNA or pBI121/siRNA+pBI121/HC-P. After 4 days, these leaves were subjected to TMV inoculation. This picture of the leaves was taken at 5 days post-inoculation. pBI, pBI121 empty vector; HC-P, pBI121/HC-Pro construct; siRNA, pBI121/siRNA construct.

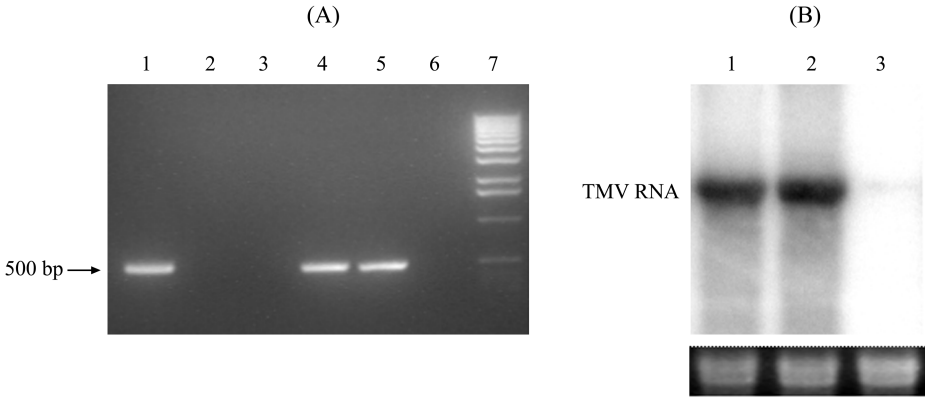

Fig. 2 RT-PCR and Northern blot analysis of the accumulation of TMV RNA in the upper leaves of *Nicotiana tabacum* plants

(A) Using specific primer pairs, the tobacco mosaic virus (TMV) can be separated from infected tobacco leaves. 1–4, samples from the leaves infiltrated with *Agrobacterium tumefaciens* cultures containing pBI121/siRNA plus pBI121/HC-Pro constructs, pBI121/siRNA construct only, pBI121/siRNA construct plus pBI121 and pBI121/HC-Pro construct plus pBI121, respectively; 5, a positive control, where the sample was from the leaves infiltrated with pBI121 only; 6, a negative control, where the RNA sample was extracted from the buffer-infiltrated, mock-inoculated leaves; 7, the mass ladder molecular marker. The 500 bp DNA of TMV 126 kDa protein is indicated by the arrow. (B) Northern blot analysis of the accumulation of TMV RNA in leaves infiltrated with *A. tumefaciens* cultures containing different constructs. 1, HC-Pro construct plus pBI121; 2, HC-Pro plus siRNA construct; 3, pBI121 plus siRNA construct. After 4 days, these leaves were inoculated with TMV. Total RNA was extracted at 15 days post-inoculation. RNA samples were fractionated by 1% agarose gel electrophoresis and hybridized with a ^{32}P-labeled DNA probe specifically for TMV genomic RNA. Ethidium bromide staining of 25S RNA was used as a loading control for the RNA gel blot (bottom panel).

with pBI121 and siRNA construct plus an empty vector. The opposite halves were inoculated with pBI121/HC-Pro plus pBI121/siRNA constructs and pBI121/HC-Pro construct plus an empty vector at the same dilution (1:1000). Our data showed that a similar number of local lesions were observed in the leaf halves of the leaves inoculated with pBI121/siRNA plus pBI121/HC-Pro constructs and pBI121/HC-Pro construct plus pBI121. No visible lesions,

Table 1 Number of lesions in a hypersensitive host

Different assays (group number)	pBI+siRNA/mock	pBI+HC-P/pBI	siRNA+HC-P/pBI	siRNA+HC-P/siRNA+pBI
1	1/0	18/24	22/23	21/1
2	0/0	23/21	19/24	18/0
3	1/0	24/21	17/23	23/0
4	0/0	22/27	21/19	20/0
5	0/0	26/23	24/20	25/2
6	0/0	23/19	25/22	19/0

Values indicate the number of necrotic local lesions (elicited by plant sap derived from the leaves of *Nicotiana tabacum* plants) per inoculated leaf in ten dependent experiments counted 5 days post-inoculation. pBI, pBI121 empty vector; siRNA, pBI121/siRNA construct; HC-P, pBI121/HC-Pro construct; mock, mock-inoculated.

or only a few local lesions, were observed in the mock-inoculated leaves and leaves inoculated with pBI121/siRNA construct plus pBI121 (**Table 1**).

Discussion

PTGS is thought to be an ancient cellular defense system acting against different molecular parasites, including transgenes, viruses and transponsons [13,20]. Thus, it is conceivable that plant viruses encode proteins able to suppress PTGS (such as HC-Pro protein of potyviruses), probably to facilitate virus infection. The HC-Pro suppression of RNA silencing induced by a GUS sense transgene eliminated the accumulation of siRNAs [21]. It has been shown that high expression levels of HC-Pro in transgenic *N. benthmiana* can partially inhibit PTGS, although the inoculated leaves display a peculiar recovery phenotype [17]. We have previously reported that the *A. tumefaciens*-mediated transient expression of siRNA inhibits TMV RNA accumulation by targeting the 126 kDa ORF, a replication-associated protein, in intact plant tissue. The interference observed is sequence-specific, as well as time- and site-dependent. The transient expression of siRNA corresponding to the TMV 126 kDa ORF can not inhibit the cucumber mosaic virus (CMV), an unrelated virus of the genus *Tobamovirus*. In this study, the transient expression of HC-Pro inhibited siRNA-mediated interference in TMV infection and diminished the antiviral silencing defense response in both systemic and hypersensitive hosts. It has been suggested that the HC-Pro suppressor activity allows replication of only a few virus particles, which is sufficient to partially counter the inhibitory effect of siRNA in the inoculated leaves. The remaining TMV RNA can probably avoid siRNA recognition and this enables it to continue moving for long distances and accumulate in distant tissues.

In animal systems, 21 nt RNA duplexes with 2 nt 3′ overhang ends are incorporated into the complexes of silencing machinery and provide sequence specificity for them [22,23]. In plants, both transgene-derived dsRNAs and double-strand replicative RNA forms of plant viruses involve a plant-encoded RNA-dependent RNA polymerase (RdRP). We assume plant RdRP also requires 21–25 nt dsRNAs as guides or primers to convert transgenic and viral mRNAs into dsRNA effectively. The HC-Pro and the RdRP complex would then compete for PTGS-generated, 21–25 nt dsRNAs. Silhavy *et al.* have shown that the P19 protein specifically binds to the synthesized 21 nt RNA oligonucleotides with 2 nt 3′ overhangs and 21–25 nt dsRNAs isolated from virus-infected plant leaves [18]. Therefore, our study suggests that the HC-Pro of PVA probably binds to the transiently expressed 21 nt dsRNAs and has acquired the ability to inhibit siRNA-directed interference in TMV infection. This inhibition effect of HC-Pro may be achieved by preventing siRNAs from interacting with either the RdRP enzyme or the RNA-induced silencing complex (RISC) and destabilizing the siRNAs. Together with other reports, this research provides an insight into the suppression of gene silencing by HC-Pro in association with the blocking of siRNA accumulation in the *Agrobacterium*-mediated transient expression system. However, how this effect is spread to distant leaves is still unknown.

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