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The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants

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RNA silencing comprises a set of sequence-specific RNA degradation pathways that occur in a wide range of eukaryotes, including animals, fungi and plants. A hallmark of RNA silencing is the presence of small interfering RNA molecules (siRNAs). The siRNAs are generated by cleavage of larger double-stranded RNAs (dsRNAs) and provide the sequence specificity for degradation of cognate RNA molecules. In plants, RNA silencing plays a key role in developmental processes and in control of virus replication. It has been shown that many plant viruses encode proteins, denoted RNA silencing suppressors, that interfere with this antiviral response. Although RNA silencing has been shown to occur in vertebrates, no relationship with inhibition of virus replication has been demonstrated to date. Here we show that the NS1 protein of human influenza A virus has an RNA silencing suppression activity in plants, similar to established RNA silencing suppressor proteins of plant viruses. In addition, NS1 was shown to be capable of binding siRNAs. The data presented here fit with a potential role for NS1 in counteracting innate antiviral responses in vertebrates by sequestering siRNAs.

INTRODUCTION

Several observations made in plants such as transgene-induced co-suppression of genes (Van der Krol *et al.*, 1990; Napoli, 1990), post-transcriptional gene silencing (English *et al.*, 1996), RNA-mediated virus resistance (Lindbo & Dougherty, 1992; de Haan *et al.*, 1992), virus-induced gene silencing, and more recently in other organisms such as quelling in fungi (Cogoni & Macino, 1997) and RNA interference (RNAi) in nematodes, insects and mammals (Elbashir *et al.*, 2001; Fire *et al.*, 1998; Tuschl *et al.*, 1999), have turned out to rely on a similar molecular process. This process, now referred to as RNA silencing, is induced by overexpressed double-stranded RNA (dsRNA) molecules and involves sequence-specific RNA degradation in the cytoplasm of eukaryotic cells (Sharp, 2001). The degradation products of this process, which is catalysed by an enzyme first identified in flies as DICER (Bernstein *et al.*, 2001), are RNAs of 21–25 nt.

Two functional classes of these molecules produced by DICER cleavage have thus far been identified: microRNAs (miRNAs) and small interfering RNAs (siRNAs). The presence of these molecules is regarded as a hallmark of RNA silencing (Hamilton & Baulcombe, 1999). In plants, miRNAs seem to be predominantly involved in targeted mRNA degradation of transcription factors that play a role in development (Llave *et al.*, 2002; Palatnik *et al.*, 2003), while siRNAs recruit specific proteins to form the RNA-induced silencing complex (RISC) and initiate sequence-specific degradation of target RNAs, such as viral RNAs (reviewed by Vaucheret & Fagard, 2001; Zamore, 2002).

The siRNA-mediated RNA silencing machinery has been suggested to play different roles in different organisms. In plants, its major function seems to be providing antiviral defence at the nucleic acid level. Indeed, *Arabidopsis* mutants exhibiting impaired RNA silencing show enhanced susceptibility to virus infection (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). In nematodes, it appears to stabilize the genome by inactivating transposable elements (Tabara *et al.*, 1999; Ketting *et al.*, 1999). In fission yeast, RNA silencing plays an important role in the regulation of the chromosome dynamics during cell division (Hall *et al.*, 2003). Interestingly, so far no naturally occurring siRNAs have been detected in mammalian cells, even though they are active in initiating RNA silencing when supplied in *trans* (McCaffrey *et al.*, 2002), a method generally used to silence endogenous genes (Elbashir *et al.*, 2001). Transfected siRNAs have been shown to protect mammalian cells efficiently against viral infection by providing sequence-specific intracellular immunity, suggesting that RNA silencing in mammalian cells can operate as an antiviral mechanism (Gitlin *et al.*, 2002).

To counteract the RNA silencing-based defence mechanism in plants, viruses encode specific proteins that have the ability to block various steps of the RNA silencing pathway (Brigneti *et al.*, 1998; Voinnet *et al.*, 1999; Beclin *et al.*, 1998; Llave *et al.*, 2000). Many of these proteins have previously been linked to 'virulence' of the virus. The HC-Pro protein of the plant-infecting potyviruses was one of the first such silencing suppressing proteins to be identified (Brigneti *et al.*, 1998). Suppression of RNA silencing by this protein is associated with a reduced accumulation of siRNAs, which is important for local RNA silencing (Llave *et al.*, 2000; Mallory *et al.*, 2001). Other viruses, such as *Cucumber mosaic virus*, express proteins that have the ability to stop the systemic silencing signal from spreading in the plant (Beclin *et al.*, 1998). Viral silencing suppressors have so far been identified in many positive-strand (Voinnet *et al.*, 1999; Brigneti *et al.*, 1998; Beclin *et al.*, 1998) and negative-strand RNA viruses (Bucher *et al.*, 2003) and DNA viruses (Voinnet *et al.*, 1999). The identification of RNA silencing suppressors has not remained limited to plant viruses, as the B2 protein of the insect-infecting *Flock house virus* has also been identified as a suppressor of RNA silencing, operating in plants as well as insect cells (Li *et al.*, 2002).

Recently, it was shown that the negative-strand RNA plant viruses *Tomato spotted wilt virus* (TSWV) and *Rice Hoja blanca virus* encode RNA silencing suppressors (Bucher *et al.*, 2003). Like these two plant viruses, related vertebrate viruses also encode non-structural proteins with assigned 'virulence' functions. Many of these proteins have been shown to interfere with innate defence responses of which the host interferon (IFN)-α/β-mediated response is the best studied (reviewed by Garcia-Sastre, 2001).

One of the most extensively studied proteins with such a 'virulence' function is the NS1 protein of influenza A virus (recently reviewed by Krug *et al.*, 2003). For this multi-functional protein, three main functional domains have been proposed. On the N-terminal part of NS1 a domain involved in translational enhancement has been mapped between aa 81 and 113 (Aragon *et al.*, 2000). This domain has been shown to interact directly with the eukaryotic translation initiation factor 4GI (eIF4G) allowing preferential translation of the influenza virus messengers. The C-terminal half constitutes the effector domain, which has been reported to inhibit mRNA processing and nuclear–cytoplasmic transport of host mRNAs (Lu *et al.*, 1995). It has been reported that NS1 interacts with a cleavage and polyadenylation specificity factor and the poly(A)-binding protein II required for 3′-end processing of cellular mRNAs (Chen *et al.*, 1999). This activity is thought to be required to prevent the maturation of host mRNAs encoding proteins with antiviral activity. Other reports suggest that the C-terminal region of the protein is mainly required for optimal dimerization of NS1 *in vivo*, which is required for its RNA-binding activity (Wang *et al.*, 2002). Additionally, NS1 contains a dsRNA-binding domain located at the N terminus of the protein (Hatada & Fukuda, 1992; Wang *et al.*, 1999). This domain has been reported to be essential for its IFN-α/β antagonistic property (Wang *et al.*, 2000). It was suggested that the mechanism of the NS1 IFN-α/β antagonistic function could be achieved by sequestering dsRNA. This would result in preventing the activation of dsRNA-dependent protein kinase (PKR) and NF-κB and thereby in inhibiting the induction of IFN-β. Interestingly, sequestering of dsRNA molecules has also been described as the mode of action of one of the best-described plant viral RNA silencing suppressors in plants, p19 of the *Tombusviruses*, which directly associates with siRNAs (Silhavy *et al.*, 2002).

DsRNAs play a central role in RNA silencing as well as in the induction of the IFN pathway. Moreover, characteristics of the established RNA silencing suppressors of plant viruses correspond to some of the described properties of influenza virus NS1. Therefore, we wanted to investigate whether influenza A virus NS1 could play a role in suppressing RNA silencing, analogous to non-structural proteins of negative-strand plant viruses. Additionally, we tested whether this function of NS1 would be effectuated by sequestering siRNAs.

METHODS

Protein expression clones, *Agrobacterium* infiltration and photography. The *Agrobacterium* clones containing the GFP, NS_S and HC-Pro expression cassettes have been described previously (Bucher *et al.*, 2003). The NS1 ORF of influenza virus A/PR/8/34 was cloned by PCR from the pRF462 plasmid provided by Ron Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands. The expression cassette was cloned in the binary vector pBIN19 and subsequently introduced into *Agrobacterium tumefaciens* (strain LBA4404). In the mutant form of NS1, NS1rb, the R35A and R38A mutations were inserted by PCR using primers EB08 (5′-**GC**GCT-TCGC**G**CAGATCAGAAATCCC-3′) and EB11 (5′-ATCAAGGAAT-GGGGCATCACCTAG-3′). *Agrobacterium* infiltration experiments and GFP imaging photography were performed as presented previously (Bucher *et al.*, 2003). Laser-scanning microscope (LSM) photographs were taken using a Zeiss LSM510 microscope.

Isolation of siRNAs, Northern blotting and non-radioactive detection. Transgenic leaf material was ground in liquid nitrogen and resuspended in 1·3 ml 2 % Sarkosyl/5 M NaCl (g leaf material)$^{-1}$. After phenol extraction, polysaccharide contaminants were precipitated with 1 vol. 3 M ammonium acetate, pH 5·2 (Sharma *et al.*, 2003). The water phase was ethanol precipitated and resuspended in TE. To remove larger RNA molecules, a PEG precipitation was performed using 5 % PEG 8000/0·5 M NaCl (final concentration) (Hamilton & Baulcombe, 1999). The supernatant containing the siRNAs was precipitated with ethanol. Twenty μg total siRNAs per sample and 10 μg total RNA of the PEG 8000 precipitate were analysed by Northern blot using a GFP-specific DIG-labelled (Boehringer) PCR product to detect the GFP siRNAs and mRNAs, respectively.

Western blot analysis, NS1 and NS1rb protein production and purification, and siRNA-binding studies. Total protein was extracted from infiltrated leaves and quantified using the Bradford assay (Bio-Rad). Ten μg total protein was analysed by Western blotting using anti-GFP and anti-rubisco (SSU) antibodies. The NS1

and NS1rb proteins were expressed in an N-terminally his-tagged form using the pQE31 vector system (Qiagen). The proteins were purified on TALON CellThru affinity columns (BD Biosiences). Synthetic double-stranded luciferase GL3 siRNAs were purchased from Qiagen. The 5′-phosphate groups of the siRNAs were removed by phosphatase treatment and replaced with ^{33}P-radiolabelled phosphate groups. Plant siRNAs were enriched, radiolabelled and purified from an 8% polyacrylamide gel using the radiolabelled luciferase siRNAs as markers. For the band-shift studies, various concentrations of NS1 or NS1rb protein were mixed with labelled siRNAs and incubated on ice for 20 min. A native 5% polyacrylamide gel was used for sample analysis (Wang *et al.*, 1999). Radiolabelled siRNAs were detected by autoradiography.

RESULTS

The influenza virus NS1 protein inhibits RNA silencing in plants

To identify RNA silencing suppressor activity of viral proteins, a standard method has been developed, which, to date, is broadly applied (Voinnet *et al.*, 2000; Johansen & Carrington, 2001). The method is based on the infiltration of two *A. tumefaciens* strains into leaves of a *Nicotiana benthamiana* plant, which is either a wild-type plant or a plant containing a GFP transgene in a silenced state. One strain carries the GFP silencing initiator reporter gene and the other the candidate silencing suppressor gene. If the candidate gene possesses silencing suppression activity, it will induce a visible boost of GFP expression in the infiltrated leaf patch. Using this method, we were able to identify the first silencing suppressors of negative-strand RNA plant viruses (Bucher *et al.*, 2003).

When infiltrating *Agrobacterium* strains carrying the GFP reporter gene in combination with one expressing the NS1 gene product, highly enhanced GFP expression was observed (Fig. 1A). The level of GFP protein expression in NS1-expressing leaves was comparable to infiltrations with the established plant viral silencing suppressor NS_S of TSWV and HC-Pro of the potyvirus *Cowpea aphid-borne mosaic virus* (CABMV) (Fig. 1B). To verify that high GFP expression was indeed due to mRNA protection rather than an enhanced translation, Northern blot analyses were performed. The high accumulation of GFP mRNAs in plant leaves demonstrated that, as for TSWV NS_S and CABMV HC-Pro, the NS1 protein protects the GFP mRNA from degradation by the RNA silencing machinery (Fig. 1C).

To elucidate further the role of NS1 in suppressing RNA silencing, siRNAs were extracted from infiltrated leaves and analysed with a GFP-specific probe (Fig. 1D). The expression of NS1 protein (Fig. 1E) drastically reduced the accumulation of GFP-specific siRNAs, indicating that the influenza virus protein directly interacts with siRNAs or inhibits siRNA production. In both cases, NS1 would interrupt the 'degradative PCR' cycle (Lipardi *et al.*, 2001; Sijen *et al.*, 2001) and thus interfere with RNA silencing.

Expression of NS1 in a *Potato virus X* vector enhances its pathogenicity

In an extensive analysis, Brigneti and co-workers (1998) showed that the expression of viral silencing suppressing proteins in a *Potato virus X* (PVX) vector drastically increased symptom severity of the virus in *N. benthamiana* plants. To test the influence of NS1 on pathogenicity, we cloned the NS1 gene in both sense and anti-sense orientation into the pGR106 PVX vector, kindly provided by the Baulcombe group. Our GFP-silenced transgenic *N. benthamiana* line (Bucher *et al.*, 2003) was then tooth-pick inoculated on a lower leaf with *Agrobacterium* containing pGR106 with NS1 in sense or anti-sense orientation and monitored for the development of viral symptoms. As shown in Fig. 2(A), the PVX vector expressing the NS1 protein (as confirmed by Western blot analysis, shown in Fig. 2D) produced severe symptoms, while the anti-sense construct typically showed mild, wild-type-like symptoms (Fig. 2A). To confirm reversal of the silenced state of the GFP transgene, systemically infected (top) leaves were monitored for GFP expression. The results showed that, on expression of NS1 from the PVX genome, local GFP-expressing patches could be observed (Fig. 2B, C).

The influenza virus NS1 protein requires a functional dsRNA-binding domain for its activity as a suppressor of RNA silencing

Since the effect of NS1 on RNA silencing could be inferred from its dsRNA-binding activity, the amino acids R35 and R38, which are essential for the dsRNA binding activity of NS1, were substituted with alanine, resulting in NS1rb (Wang *et al.*, 1999). *Agrobacterium* infiltration experiments were repeated with this mutant form of NS1 and showed that this protein was unable to enhance GFP expression (Fig. 1A, B). Northern blot analysis of the RNA extracted from the infiltrated patch showed that NS1rb did not protect the GFP mRNA from degradation (Fig. 1C) indicating that the mutant protein was incapable of inhibiting RNA silencing. Ongoing, active RNA silencing in the presence of mutant NS1 was further demonstrated by the presence of GFP-specific siRNAs (Fig. 1D). Expression of NS1 and NS1rb was confirmed by Western blot analysis (Fig. 1E).

The influenza virus NS1 protein binds siRNAs

NS1 has previously been reported to bind dsRNA molecules as short as 50–140 nt (Wang *et al.*, 1999). Combining the observation that NS1 is involved in RNA silencing suppression in plants with the fact that it requires a functional dsRNA-binding domain, we examined whether NS1 could exert this function by binding siRNAs.

For this purpose, gel-shift experiments were performed with purified NS1 protein produced in *E. coli*. As shown in Fig. 3(A), the NS1 protein was able to bind radiolabelled synthetic 21 bp siRNAs resulting in band shifting. Similarly, NS1 was capable of binding radiolabelled siRNAs extracted from plants (Fig. 3B).

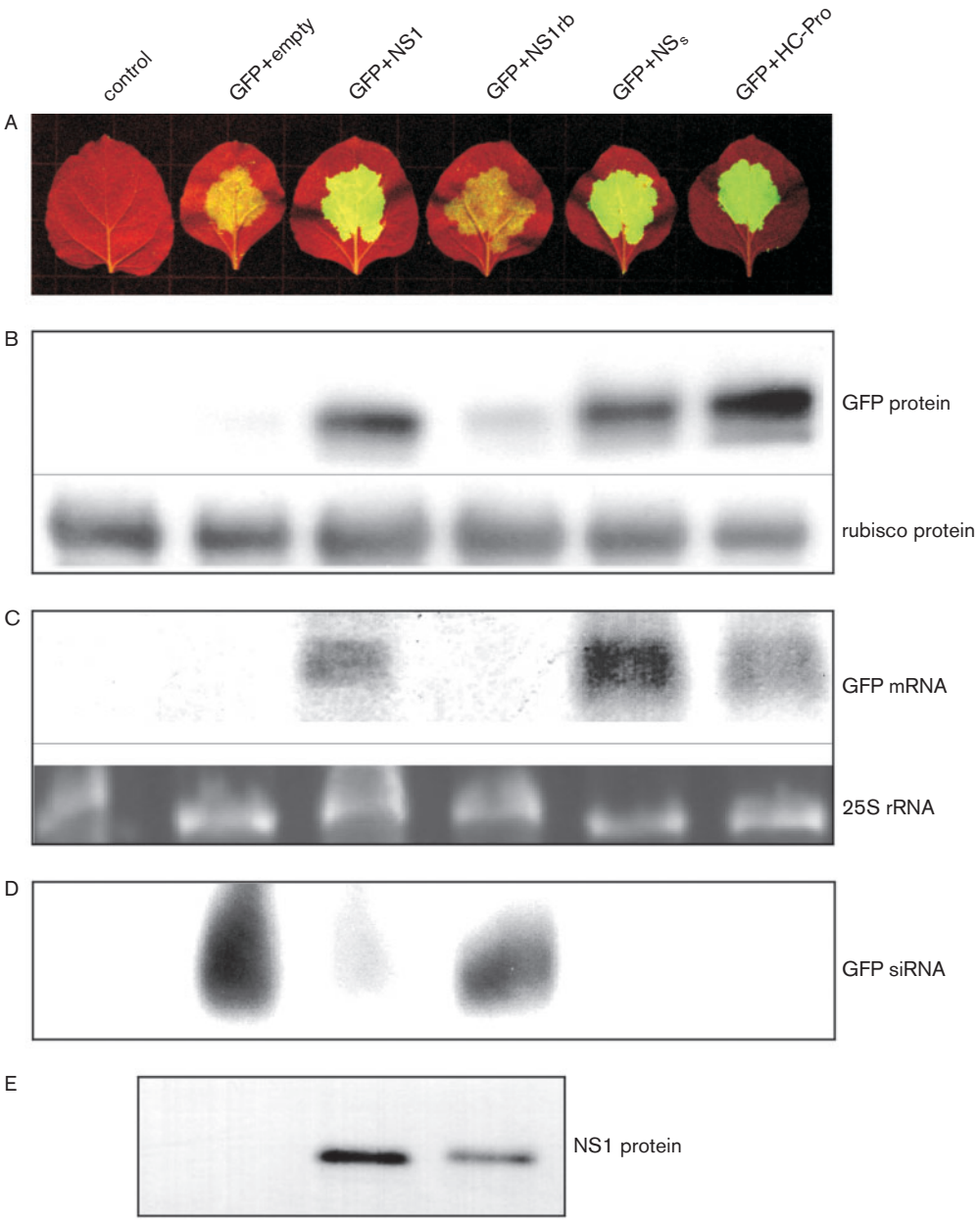

Fig. 1. Effect of wild-type NS1 and mutant NS1rb on RNA silencing of GFP in *Nicotiana benthamiana* leaves. (A) UV photography of *Agrobacterium*-infiltrated leaves. From left to right: non-infiltrated wild-type, and GFP expression constructs co-infiltrated with an empty binary vector, the influenza virus NS1 gene construct, the NS1rb mutant, NS_S and HC-Pro, respectively. (B) Quantitative Western blot analysis performed on total protein extracted from infiltrated sectors of the leaf using anti-GFP antibodies. Rubisco protein abundance in these green leaves was used as a loading control and was visualized using anti-rubisco (SSU) antibodies. (C) Northern blot analyses of mRNA purified from infiltrated leaf sectors, probed with a DIG-labelled GFP-specific PCR fragment. Ethidium bromide staining of the same gel shows the 25S rRNA as a loading control. (D) GFP siRNAs extracted from infiltrated leaf sectors and detected using a DIG-labelled GFP-specific probe. (E) Expression of NS1 and NS1rb protein analysed by Western blot using NS1-specific antibodies.

Since NS1rb lost its capacity to suppress RNA silencing, we tested whether this mutant was deficient in binding siRNAs. His-tagged NS1rb protein was purified from *E. coli* and tested using the same band-shift experiment as wt NS1 (Fig. 3A). The results showed that the NS1 protein with mutations in the RNA-binding domain was no longer capable of binding siRNAs, whether synthetically produced or isolated from plants (Fig. 3A, B).

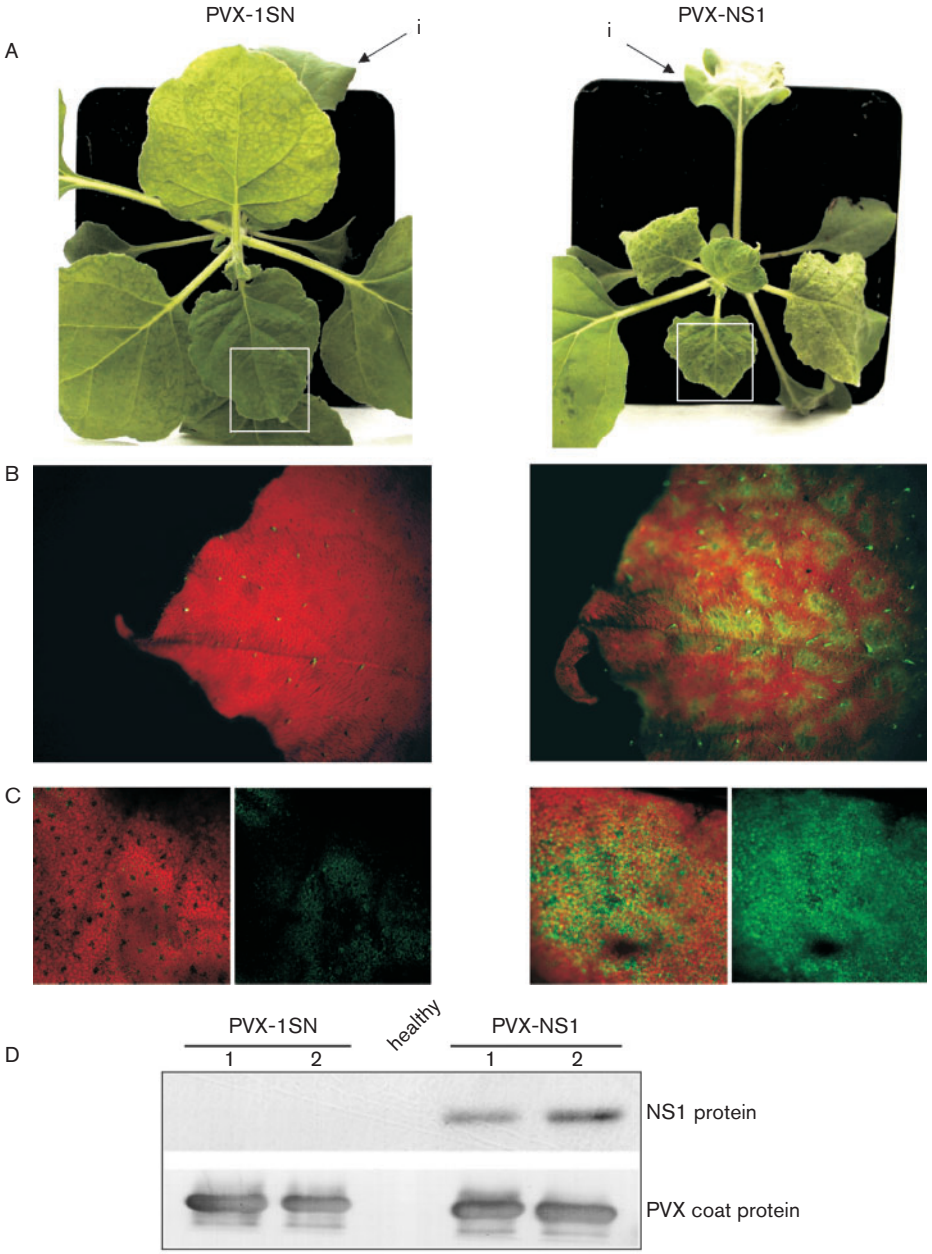

Fig. 2. Analysis of plants infected with PVX carrying the NS1 gene. (A) Symptoms caused by the infection with PVX-1SN (NS1 cloned in anti-sense orientation) and PVX-NS1 (NS1 cloned in sense orientation) in *N. benthamiana* plants 6 days after inoculation. The inoculation sites (i) are indicated by arrows. (B) UV photographs of a close-up of systemically infected GFP-silenced *N. benthamiana* leaves (section indicated by squares in panel A) infected with either PVX-1SN or PVX-NS1. (C) LSM pictures of a fluorescent patch caused by PVX-NS1 compared with PVX-1SN (left panel: red and green overlay; right panel: green only). The same light intensities were used for both pictures. (D) Western blot analysis of leaves originating from two systemically infected plants using NS1- and PVX coat protein-specific antibodies.

DISCUSSION

Many animal and plant viruses carry genes that have been described as being involved in virulence. Mutation or removal of these genes generally resulted in an attenuated phenotype of the virus (Garcia-Sastre *et al.*, 1998; Bergmann *et al.*, 2000; Qu & Morris, 2002). In plant viruses, many of these genes are involved in suppression of the RNA silencing machinery of the host (reviewed, for example, by Li & Ding, 2001). We have also recently shown that negative-strand RNA plant viruses of the *Tospovirus* and *Tenuivirus* genera carry such suppressors of RNA silencing (Bucher *et al.*, 2003). Negative-strand viruses form a minority among the

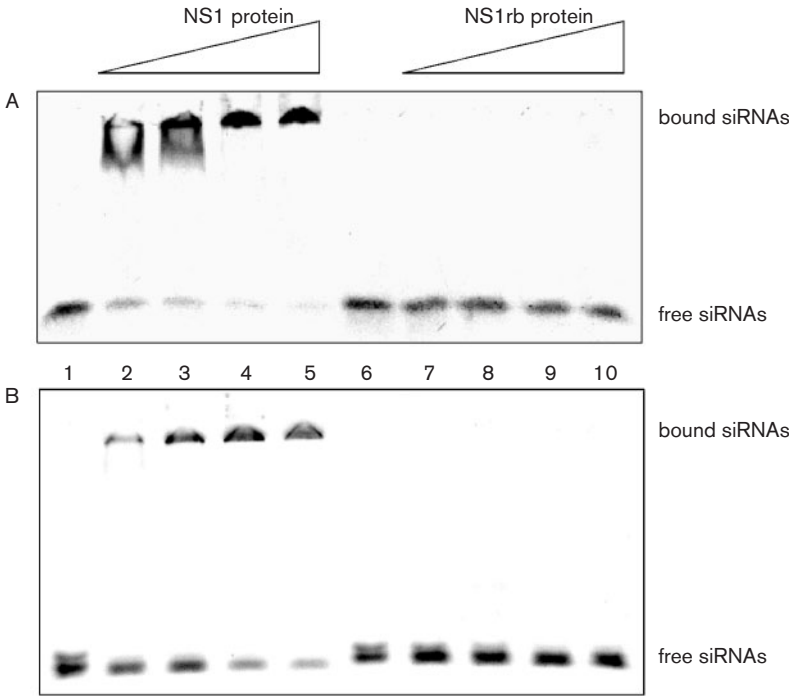

Fig. 3. Gel retardation studies of siRNAs binding to increasing amounts of NS1 protein. (A) Radiolabelled synthetic siRNAs (2 pM) incubated with 0, 25, 50, 100 and 200 pM purified NS1 (lanes 1–5) and NS1rb (lanes 6–10), visualized by radiography after native gel electrophoresis. (B) Radiolabelled purified plant siRNAs incubated under the same conditions.

plant viruses, but are much more common in animal hosts (Van Regenmortel *et al.*, 2000).

We have demonstrated here that, as for established plant viral RNA silencing suppressors such as HC-Pro or NS_S, the influenza A virus protein NS1 enhances expression of an *Agrobacterium*-delivered reporter protein construct by protecting its mRNAs from degradation. Concomitantly, siRNA production is drastically reduced, which indicates that NS1 interferes with the plant RNA silencing machinery. Additionally, here and in Delgadillo *et al.* (2004) (accompanying paper) it has been shown that expression of NS1 from a PVX viral vector results in strong enhancement of symptoms, a phenomenon often observed when expressing RNA silencing suppressors in plants (Brigneti *et al.*, 1998). Additionally, we have shown that the expression of NS1 in a PVX background leads to some local suppression of an established RNA silencing state in transgenic GFP-silenced plants.

The siRNA-binding capability of NS1 indicates that it may sequester free siRNAs during infection. By doing so it prevents the siRNAs from being incorporated into the RISC complex and taking part in the generation of longer dsRNA molecules and subsequent DICER/RISC-mediated degradative PCR of (viral) RNAs. In previous research, NS1 has been shown to bind longer dsRNAs (Wang *et al.*, 1999), although it is less efficient in binding to dsRNAs than other cellular dsRNA-binding proteins (Krug *et al.*, 2003). Here we have shown that NS1 has a high affinity for siRNAs. It would be interesting to investigate whether NS1 has a higher affinity for the class of larger siRNAs (24–26 nt), since independent research by Delgadillo *et al.* (2004) shows that this size class is most affected by NS1 action. In plants, the smaller-sized class of siRNAs (21–22 nt) has been implicated in suppression of local RNA silencing (RISC action), while the larger-sized class seems to be involved in systemic silencing and methylation status of homologous DNA (Hamilton *et al.*, 2002). As shown in Fig. 1, NS1 has a clear effect on local RNA silencing leading to re-emergence of GFP expression, while Delgadillo *et al.* (2004) have also shown an effect on the suppression of systemic silencing.

It can be argued that specific siRNA binding by NS1 merely reflects its general capacity to bind any dsRNA. Indeed, a recent report showed that several dsRNA-binding proteins were capable of acting as RNA silencing suppressors in plants (Lichner *et al.*, 2003). It was suggested that they act by sequestering (larger) dsRNA molecules and hiding them from the silencing machinery. In addition to the NS1 protein, we have expressed the NSP5 protein of rotavirus, another viral dsRNA-binding protein (Vende *et al.*, 2002), in the *Agrobacterium* infiltration assay. However, expression of this protein did not result in RNA silencing suppression (results not shown) and may suggest that RNA silencing suppression is not a general feature of dsRNA-binding proteins.

Recent elegant work by the Burgyàn group has demonstrated that the P19 protein of the plant-infecting *Tombusviruses* suppresses RNA silencing in plants by sequestering siRNAs through direct binding (Silhavy *et al.*, 2002). Here we have shown that, as well as its previously demonstrated activity of binding larger dsRNA molecules, NS1, like P19, efficiently binds siRNAs, either synthesized synthetically

or isolated from plants. The dsRNA-binding motif in NS1 is required for binding siRNAs, as it is for its RNA silencing suppression activity in plants.

It may be assumed that, as in plants, NS1 binds siRNAs and longer dsRNAs in its natural host. In plants, the involvement of siRNAs is essential for virus inhibition through RNA silencing. In animals, larger dsRNA molecules (Williams, 1999) as well as siRNAs (Bridge *et al.*, 2003; Sledz *et al.*, 2003) have been demonstrated to be involved in the sequence-unspecific initiation of the IFN-mediated innate antiviral response. It has been demonstrated that dsRNA triggers the secretion of IFNs, which subsequently induce the production of PKR, 2′-5′-oligoadenylate synthetases and many more proteins with proposed antiviral activities (Rebouillat & Hovanessian, 1999; Williams, 1999; Kaufman, 1999). Until recently, it was assumed that only longer stretches of dsRNA could induce the IFN response. However, recent reports suggest that short hairpin RNAs and siRNAs, designed to initiate RNA interference, can also trigger the IFN response (Bridge *et al.*, 2003; Sledz *et al.*, 2003).

As yet, it cannot be excluded that the function of NS1 in mammalian cells may be limited to sequestering siRNA and larger dsRNA molecules from detection by the IFN-α/β response and PKR (Garcia-Sastre, 2001). However, siRNA molecules, when transfected into mammalian cells, have been demonstrated to inhibit virus replication in a sequence-specific manner (Gitlin *et al.*, 2002; Andino, 2003), suggesting an active sequence-specific RNA silencing machinery, which, as in plants and insects, can act as an antiviral response in mammalian cells. Taking this assumption further, this would imply that, next to or underlying the IFN-α/β response, RNA silencing may play a role in antiviral defence in mammals. It is interesting to note that the amino acids involved in the IFN antagonistic properties of NS1 in mammalian cells coincide with those essential for RNA silencing suppression in plants.

Taken together, we have demonstrated here that NS1 suppresses RNA silencing in plants by binding siRNAs. Our data thus suggest that RNA silencing may represent an important inhibitory function of virus infection that is counteracted by specific viral proteins, not only in plants, where it is well established, but also in animals. Clearly, to prove this final assumption, it will be of great importance to investigate the effects of NS1 on siRNA- and dsRNA-induced RNA silencing in mammalian systems using the recently established experimental protocols (Gitlin *et al.*, 2002; McCaffrey *et al.*, 2002).

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NOTE ADDED IN PROOF

Two weeks after the acceptance of this paper, a paper confirming our conclusions was submitted to and accepted for *Proc Natl Acad Sci U S A*.

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