

Short Communication

Binding of small interfering RNA molecules is crucial for RNA interference suppressor activity of rice hoja blanca virus NS3 in plants

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The NS3 protein of rice hoja blanca virus represents a viral suppressor of RNA interference (RNAi) that sequesters small interfering (si)RNAs *in vitro*. To determine whether this siRNA binding property is the critical determinant for the suppressor activity of NS3, NS3 was altered by alanine point mutations and the resulting mutant proteins were tested for both siRNA binding ability and RNAi suppressor activity in plants. Alanine substitutions of lysine residues at positions 173–175 resulted in mutant proteins that lost both their affinity for siRNAs and their RNAi suppressor activity *in planta*. This indicates that siRNA binding of NS3 is indeed essential for the suppressor function of NS3 and that residues at positions 173–175 are involved in the siRNA binding and suppressor activities.

As a response to antiviral RNA interference (RNAi), plant viruses encode antagonistic proteins, often referred to as RNAi suppressor proteins, which counteract or evade this host defence mechanism (reviewed by Ding & Voinnet, 2007; Voinnet, 2005). A typical suppressor action adopted by plant viruses is the binding to double stranded (ds)RNA molecules, in either a size-specific manner to 21–26 nucleotide (nt) small interfering (si)RNA or a non-size-specific manner (Lakatos *et al.*, 2006; Merai *et al.*, 2006). The 21 nt siRNA molecules play important roles in different RNAi pathways (Brodersen & Voinnet, 2006; Vaucheret, 2006). One strand of the siRNA duplex is incorporated in the RNA-induced silencing complex (RISC) to guide the sequence-specific recognition of complementary targets, resulting in cleavage (Tomari & Zamore, 2005). By sequestering siRNAs, a considerable number of viral suppressors remove these molecules from the RNAi pathway, thereby inhibiting RISC assembly (Lakatos *et al.*, 2006). Among plant viruses, the tenuiviruses are unusual because they replicate both in their insect vector (leafhopper) (Falk & Tsai, 1998) and plant host (rice). The RNAi

suppressor protein (NS3) of rice hoja blanca virus (RHBV) (Bucher *et al.*, 2003) inhibits antiviral RNAi in both plant and insect cells and efficiently binds 21 nt siRNA *in vitro* (Hemmes *et al.*, 2007). As NS3 also inhibits induced RNAi in mammalian cells (Schnettler *et al.*, 2008), it is likely that binding siRNAs is the crucial biochemical activity of this protein for suppression of RNAi. If this is the case, loss of RNAi suppressor activity should coincide with loss of binding affinity to siRNA molecules. To test this, selected amino acids in the NS3 sequence were altered by point mutagenesis and the resulting mutant proteins were monitored for RNAi suppressor activity in plants and for siRNA binding capacity *in vitro*. Using CLUSTAL_X, sequences of the available tenuiviral NS3 proteins (rice stripe virus, maize stripe virus, RHBV, echinocloa hoja blanca virus, uruchloa hoja blanca virus and rice grassy stunt virus) were aligned to identify regions or sequence motifs that are potentially important for RNA binding. A common feature of amino acids that interact with RNA molecules in a broad range of proteins is that they are polar and positively charged (Haasnoot *et al.*, 2007; Hartman *et al.*, 2004; van Rij *et al.*, 2006; Wang *et al.*, 1999). Several conserved areas were found in the alignment, of which only a few matched with the requirement to be conserved, surface-exposed and containing positively charged residues (K, R or H) (Fig. 1a and b). One of these regions (residues 106–114; region 1) is located centrally in the protein; the other (residues 167–176; region 2) is near the C terminus. Region 2 contains a marked cluster of three positively charged lysines (at residues 173–175).

[†]Dedicated to Rob Goldbach who sadly passed away on 7 April 2009.

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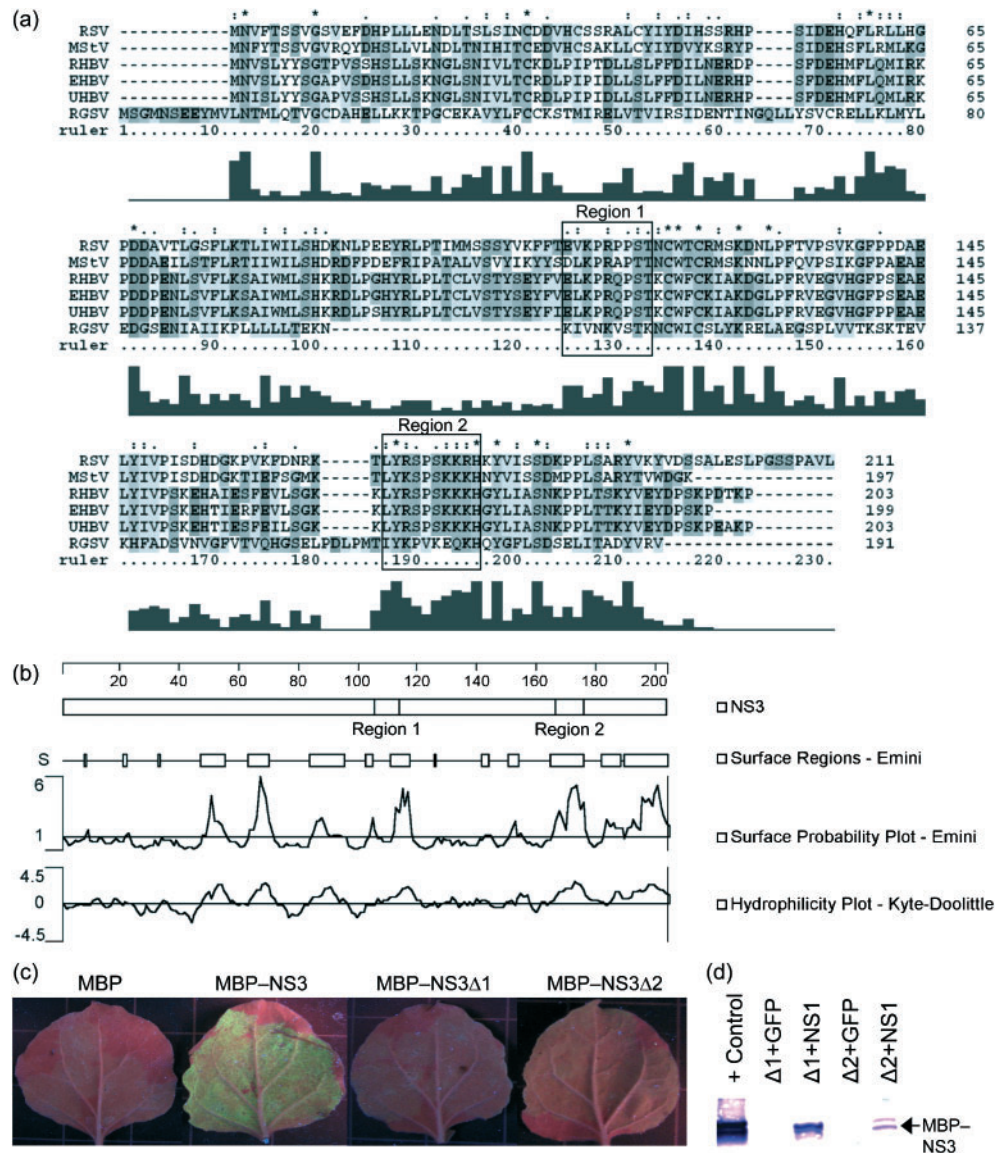


Fig. 1. Analysis of the tenuivirus NS3-like proteins. (a) Sequence alignment of six NS3-like proteins originating from rice stripe virus (RSV; NS3), maize stripe virus (MSStV; p3), RHBV (NS3), echinocloa hoja blanca virus (EHBV; pv3), urochloa hoja blanca virus (UHBV; pv3) and rice grassy stunt virus (RGSV; p5), using CLUSTAL_x. Amino acid identity: identical amino acids, conserved substitutions and semi-conserved substitutions are indicated by asterisks (*), double dots (:) and dots (.), respectively. (b) Surface probability plot and hydrophilicity plot of the RHBV NS3 protein, determined using Protean (DNASTAR). (c) RNAi suppression assay on *N. benthamiana* leaves. GFP expression was visualized 5 days post-infiltration in leaves co-infiltrated with *Agrobacterium tumefaciens* harbouring binary vectors encoding mGFP and MBP, MBP-NS3, MBP-NS3Δ1 or MBP-NS3Δ2. (d) Expression of MBP-NS3Δ1 and MBP-NS3Δ2 was confirmed by Western blot analysis of leaves co-infiltrated with NS1 of influenza A virus, using an MBP-specific antibody. As a positive control, bacterial purified MBP-NS3 was used.

To test the potential functionality of both regions in RNAi suppressor activity and siRNA binding capacity, regions 1 or 2 were entirely removed in maltose-binding (MBP)-NS3 fusion constructs (Hemmes *et al.*, 2007; Karimi *et al.*, 2002) and these were expressed using the pk2GW7 plant expression vector (Karimi *et al.*, 2002). Both deletion mutants (NS3Δ1 and NS3Δ2) were tested for their RNAi suppressor activity in *Nicotiana benthamiana* using the

Agrobacterium transient transformation assay (ATTA) with mGFP as a reporter gene, as described previously (Bucher *et al.*, 2003). A drastic decrease in the fluorescence level of green fluorescent protein (GFP) was observed for both NS3Δ1 and NS3Δ2 comparable to the negative control MBP (Fig. 1c). Western blot analysis of infiltrated leaf extracts, using an MBP-specific antibody, determined that the observed lack of RNAi suppression activity was not due

to poor expression or protein instability of these deletion mutants (Fig. 1d). Both mutants were readily detected in leaves but only upon co-infiltration with an active RNAi suppressor, e.g. NS1 of influenza virus A (Bucher *et al.*, 2004) (Fig. 1d), probably because expression of inactive RNAi suppressors is hampered by RNAi. Hence, the conclusion could be drawn that both deleted regions 1 and 2 were crucially involved in the RNAi suppressor activity of NS3 *in planta*.

Both regions were analysed further by single alanine substitutions. Based on surface probability, conservation and positive charge, five single amino acid substitutions were made in region 1 (E110A, L111A, K112A, P113A and R114A) and three in region 2 (K173A, K174A and K175A) using standard PCR technology. Single point mutations at positions 111 and 114 (L111A and R114A, respectively) repeatedly failed to yield a stable mutant; hence a total of six single alanine mutants were tested for loss of RNAi suppressor activity and siRNA binding. Five days post-infiltration, wild-type levels of GFP were observed for all mutants in three independent repeats, indicating that no single residue in region 1 or 2 was critical for RNAi suppressor activity *in planta* (Fig. 2a, upper panel). Northern blot analysis showing increased GFP-specific mRNA confirmed their suppression activity (Fig. 2a, central panel).

GFP-specific siRNAs, extracted and enriched as described previously (Bucher *et al.*, 2003), were present in each sample and, taking the loading control into account, no significant differences in siRNA concentration were

detected compared to the positive control (wild-type NS3) (Fig. 2a, lower panel).

In line with this result, all single alanine substitution mutants retained a high affinity for siRNA molecules, indicated by a low dissociation constant (K_d): E110A, 133 ± 9.2 nM; K112A, 2.8 ± 0.3 nM; P113A, 136 ± 8.3 nM; K173A, 7.7 ± 2.0 nM; K174A, 12.4 ± 3.2 nM; K175A, 10.0 ± 2.1 nM [data taken from three independent electrophoretic mobility shift assays (EMSAs), data not shown]. As controls, MBP-NS3 (K_d 4.7 ± 0.5 nM; see Fig. 3a) and MBP were used. MBP showed no binding to siRNAs at all tested concentrations (data not shown).

Clustered basic amino acids have been shown to be important for the suppressor activity of influenza A virus NS1, tombusvirus p19 protein and *Drosophila* C virus (DCV) 1A (Bucher *et al.*, 2004; Chu *et al.*, 2000; Haasnoot *et al.*, 2007; van Rij *et al.*, 2006; Vargason *et al.*, 2003; Wang *et al.*, 1999) and single mutations often proved insufficient to destroy RNA binding ability (Wang *et al.*, 1999). Therefore, double and triple alanine substitutions were considered, especially for the clustered positively charged residues. Region 2 of NS3 encompasses such a cluster, i.e. three conserved lysines at positions 173–175 (Fig. 1a), which shares high similarities with the DCV 1A protein, shown to lose its RNAi suppressor activity and the linked affinity to long dsRNA if a double mutation is created (K73A/K74A) (van Rij *et al.*, 2006).

Double and triple alanine substitutions were introduced by PCR (primer sequences available on request), resulting in

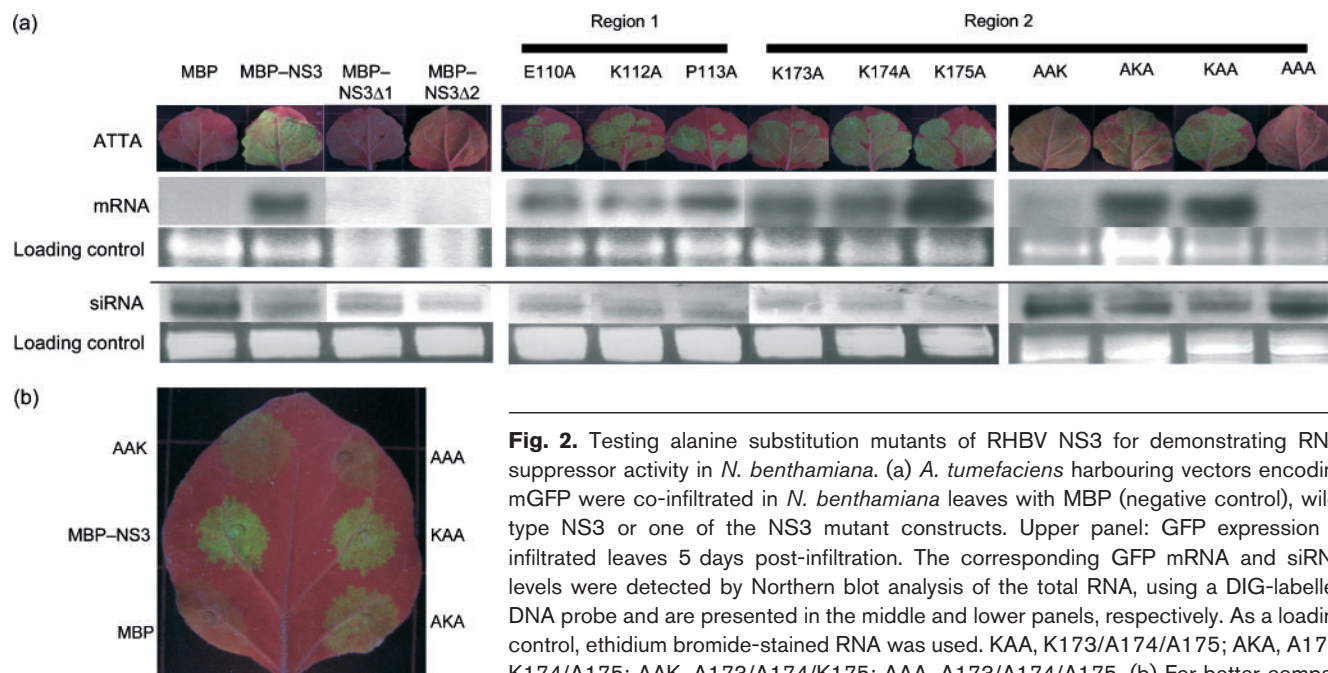


Fig. 2. Testing alanine substitution mutants of RHBV NS3 for demonstrating RNAi suppressor activity in *N. benthamiana*. (a) *A. tumefaciens* harbouring vectors encoding mGFP were co-infiltrated in *N. benthamiana* leaves with MBP (negative control), wild-type NS3 or one of the NS3 mutant constructs. Upper panel: GFP expression in infiltrated leaves 5 days post-infiltration. The corresponding GFP mRNA and siRNA levels were detected by Northern blot analysis of the total RNA, using a DIG-labelled DNA probe and are presented in the middle and lower panels, respectively. As a loading control, ethidium bromide-stained RNA was used. KAA, K173/A174/A175; AKA, A173/K174/A175; AAK, A173/A174/K175; AAA, A173/A174/A175. (b) For better comparison, the various NS3 constructs and controls were co-infiltrated in different patches within a single leaf and GFP expression was visualized 5 days post-infiltration.

four possible mutant proteins (AAK, A173/A174/K175; KAA, K173/A174/A175A; AKA, A173/K174/A175; AAA, A173/A174/A175). By testing these mutants for RNAi suppressor activity in *N. benthamiana* leaves, it was found that double mutant AAK and triple mutant AAA, but not the two other double mutants (KAA and AKA), were defective in RNAi suppression, as revealed by lack of GFP expression (Fig. 2a, upper panel and Fig. 2b) and absence of GFP mRNA (Fig. 2a, central panel). Regarding siRNA binding ability, wild-type protein showed a K_d in the order of wild-type protein (56.9 ± 8.3 nM and 10.8 ± 1.2 nM, respectively; Fig. 3b, c and f), while mutants AAK and AAA completely lost their siRNA binding capacity ($K_d > 750$ nM; Fig. 3d–f).

These findings indicate that binding of siRNA is an important activity of RHBV NS3 to counteract antiviral RNAi in plants. RHBV multiplies in both its plant host (rice) and its insect vector (plant hopper) (Falk & Tsai, 1998; Ramirez *et al.*, 1992); thus it must be able to counteract antiviral RNAi in both. It is therefore not

surprising that NS3 acts by sequestering siRNAs, key molecules of the RNAi pathway in a variety of organisms.

In all, the presented data unequivocally demonstrate that binding capacity of siRNA, which can be disrupted with double and triple mutations in a triple lysine motif in region 2, is essential for NS3 suppressor activity in plants. Prediction of the secondary structure, using PSIPRED, revealed only a small and similar change for all four mutants (data not shown). Hence, it is very likely that the triple K motif at positions 173–175 is specifically involved in siRNA binding, rather than gross alterations in the NS3 folding crippling the protein upon the amino acid substitutions. Since clustered positively charged amino acids have been found to be important for the activity of other dsRNA-binding viral RNAi suppressors (Bucher *et al.*, 2004; Chu *et al.*, 2000; Haasnoot *et al.*, 2007; van Rij *et al.*, 2006; Wang *et al.*, 1999), it is tempting to assume that the triple K motif represents a key part of the siRNA binding domain. Solving the crystal structure of the NS3 protein may confirm this notion.

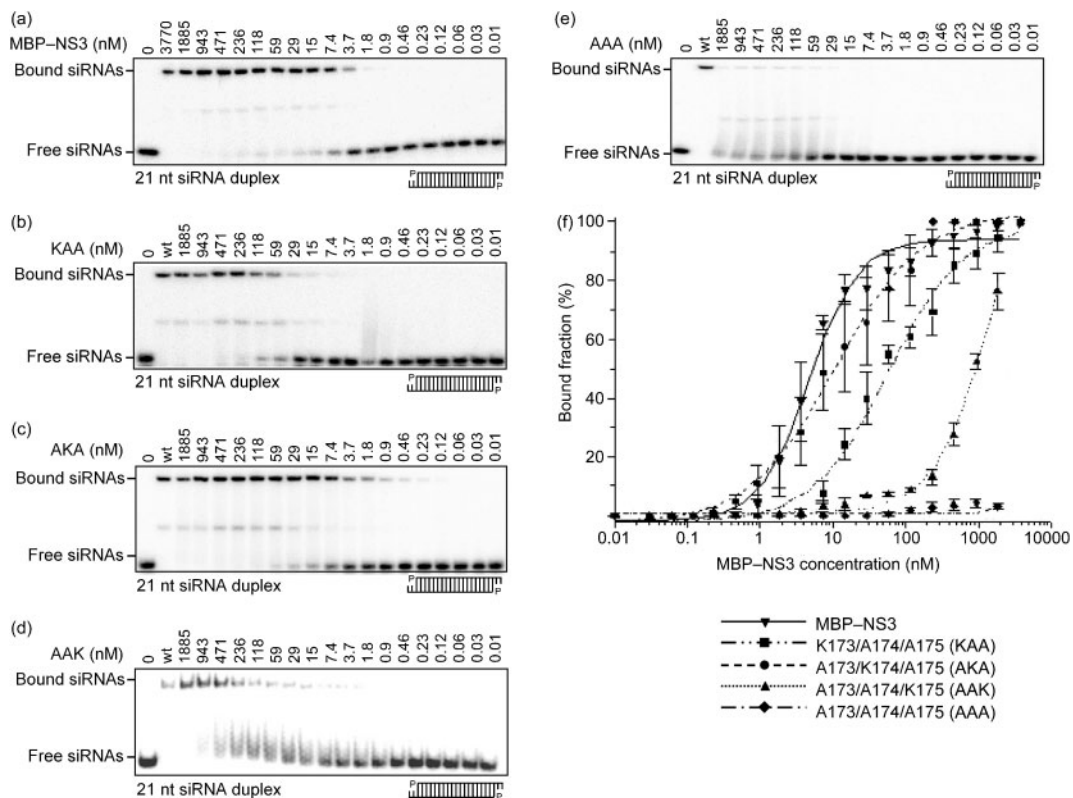


Fig. 3. Affinity of MBP-NS3 mutated proteins for siRNA duplexes. A dilution series (0.01–3770 nM) of bacterially expressed and purified N-terminally fused NS3 mutated proteins was incubated with 100 pM ^{32}P -labelled 21 nt siRNA duplexes in binding buffer [100 mM NaCl, 20 mM Tris/HCl pH 7.4, 1 mM DTT, 2.5 mM MgCl_2 , 10% (v/v) glycerol] for 20 min, then loaded onto a 5% native acrylamide gel. The gel was exposed overnight to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphor imager, Amersham Biosciences); bands were quantified using Genius Image Analyser software (Syngene). As a control, wild-type MBP-NS3 was used (a). Mutated proteins are: (b) KAA, (c) AKA, (d) AAK and (e) AAA. (f) The K_d for the different MBP-NS3 proteins was determined by plotting the bound RNA fraction as function of the MBP-NS3 concentration. The K_d of the different MBP-NS3 proteins represents the protein concentration where 50% of the siRNA was bound.

Single alanine substitutions of the investigated residues, either in region 1 or 2, did not affect RNAi suppressor activity in the infiltrated *N. benthamiana* leaves and these alterations did not lead to a drastic decrease in the binding affinity for siRNA either. This is consistent with observations for other RNAi suppressor proteins, known to act through dsRNA binding, including NS1 of influenza A virus (Bucher *et al.*, 2004; Wang *et al.*, 1999), VP35 of ebola virus (Haasnoot *et al.*, 2007) and 1A of DCV (van Rij *et al.*, 2006), which all required at least a double mutation to remove their RNAi suppressor activity.

It should be noted that, based on the results of the EMSA, the K_d for siRNAs of NS3 proteins with single alanine substitutions or AAK and KAA substitutions ranged from 3 to 130 nM, which is higher than that of wild-type NS3 (4.7 ± 0.5 nM). However, even a protein with a K_d of 130 nM is considered to have a significant affinity for the target molecule. These results, together with the fact that these mutants still significantly suppress RNAi in plants, may indicate that the K_d of plant pre-RISC (or RISC loading) complexes for siRNAs is higher than 130 nM. Alternatively, in the chosen experimental setup, the NS3 mutant proteins may have accumulated to high levels compared with RISC (loading) complexes, making proteins with a relatively low affinity sufficiently strong RNAi suppressors. In this context, it would be interesting to see how these mutations behave in natural infections, but at present this is not possible due to the lack of a reverse genetics system for tenuiviruses. Our mutational analysis has also revealed at least one additional region (region 1; Fig. 1) that may be involved in the functionality of NS3. Deletion of this region also caused loss of suppressor activity *in planta* and further research is needed to elucidate the significance of this finding.

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