Analysis of *Tomato spotted wilt virus* NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppression

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SUMMARY

Recently, *Tomato spotted wilt virus* (TSWV) nonstructural protein NSs has been identified unambiguously as an avirulence (Avr) determinant for *Tomato spotted wilt* (*Tsw*)-based resistance. The observation that NSs from two natural resistance-breaking isolates had lost RNA silencing suppressor (RSS) activity and Avr suggested a link between the two functions. To test this, a large set of NSs mutants was generated by alanine substitutions in NSs from resistance-inducing wild-type strains (NSs^{RI}), amino acid reversions in NSs from resistance-breaking strains (NSs^{RB}), domain deletions and swapping. Testing these mutants for their ability to suppress green fluorescent protein (GFP) silencing and to trigger a *Tsw*-mediated hypersensitive response (HR) revealed that the two functions can be separated. Changes in the N-terminal domain were found to be detrimental for both activities and indicated the importance of this domain, additionally supported by domain swapping between NSs^{RI} and NSs^{RB}. Swapping domains between the closely related *Tospovirus Groundnut ringspot virus* (GRSV) NSs and TSWV NSs^{RI} showed that Avr functionality could not simply be transferred between species. Although deletion of the C-terminal domain rendered NSs completely dysfunctional, only a few single-amino-acid mutations in the C-terminus affected both functions. Mutation of a GW/WG motif (position 17/18) rendered NSs completely dysfunctional for RSS and Avr activity, and indicated a putative interaction between NSs and Argonaute 1 (AGO1), and its importance in TSWV virulence and viral counter defence against RNA interference.

INTRODUCTION

RNA silencing (also named RNA interference, RNAi) is part of the innate immune response in plants against viral invasion. It is triggered by viral double-stranded RNA (dsRNA), derived from secondary folding structures or replicative intermediates, that is cleaved in the cytoplasm by a host Dicer-like (DCL) protein into

short interfering RNAs (siRNAs) of 21–24 nucleotides in length (Ding and Voinnet, 2007). Upon unwinding of siRNAs, one of the strands (guide strand) is uploaded into the RNA-induced silencing complex (RISC), whereas the other strand is destroyed. Activated RISC surveys the cytoplasm for the presence of complementary (viral) RNA target molecules, and degrades these using Argonaute (AGO) slicing activity. Viruses evade or suppress this pathway to achieve a successful infection in the plant host. Most viruses do this by encoding RNA silencing suppressors (RSSs), proteins that interfere at various steps in the RNA silencing pathway (Díaz-Pendón and Ding, 2008). Many plant RNA viruses code for an RSS that exerts its function by sequestering long dsRNA or siRNAs, and thereby preventing their cleavage by DCL and their uploading into RISC, respectively. Although other modes of interference [at the level of AGO1, DCL and RNA-dependent RNA polymerase (RdR)] have also been reported (Giner *et al.*, 2010; Incarbone and Dunoyer, 2013; Zhang *et al.*, 2006), all prevent viral RNA target molecules from becoming degraded by RISC. For *Tomato spotted wilt virus* (TSWV), representative of the plant-infecting bunyaviruses (Kormelink *et al.*, 2011), the NSs protein has been identified as an RSS (Bucher *et al.*, 2003; Takeda *et al.*, 2002), and most probably exerts this activity by sequestering long dsRNA and small siRNAs (Schnettler *et al.*, 2010). In addition to its RSS activity, NSs enhances the translation of viral transcripts, which appears most strongly in the additional presence of the viral nucleoprotein (N). It is postulated that NSs is able to do this as a kind of functional equivalence to a poly(A) tail-binding protein (PABP) by binding to the dsRNA hairpin structure predicted from the 3'-untranslated region of viral transcripts and encoded by the intergenic region of the ambisense RNA elements (Geerts-Dimitriadou *et al.*, 2012).

TSWV is also prone to a second line of defence in plants that is based on dominant resistance (*R*) genes, and is also referred to as effector-triggered immunity (ETI) (Jones and Dangl, 2006). During this defence mechanism, a host *R*-gene product (in-)directly perceives the virus by one of its (effector) proteins, which activates a programmed cell death response visualized by a hypersensitive response (HR). The resistance model explaining the indirect recognition of avirulence (Avr) proteins is often referred to as the guard model or decoy model, in which the *R*-gene product guards a host factor (functional or decoy) which,

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on interaction with the Avr protein, triggers *R* gene-mediated resistance (Moffett, 2009). Two dominant *R* genes are available for commercial breeding against TSWV, both belonging to the nucleotide-binding site-leucine-rich repeat (NBS-LRR) class of *R* genes (de Ronde *et al.*, 2013), namely the *SW5b* gene from tomato and the *Tomato spotted wilt (Tsw)* gene from pepper. Although, in the past, conflicting papers have appeared on the identification of the Avr gene from TSWV on recognition by the *Tsw* gene product causing an HR in pepper (Lovato *et al.*, 2008; Margaria *et al.*, 2007), recently, we have unambiguously identified NSs as the Avr determinant for *Tsw*-based resistance (de Ronde *et al.*, 2013). The analysis of two natural resistance-breaking (RB) field isolates and the characterization of their NSs proteins showed that, for these isolates, the NSs protein lacks both RSS and Avr activity, indicating a putative link between the two activities (de Ronde *et al.*, 2013).

Here, we have generated and tested a large set of NSs mutants for their ability to suppress green fluorescent protein (GFP) silencing and to trigger *Tsw*-mediated HR. The results indicate that both functions can be individually disrupted, although the N-terminal part of NSs seems to hold the most important sequences for both functions.

RESULTS

Generation of NSs mutants

To test whether the RSS activity and elicitation of *Tsw*-mediated HR were indeed tightly linked and, furthermore, to identify the role of certain (conserved) amino acids, motifs and domains in NSs for either one of these functions, a large NSs mutant screen was performed. To this end, three series of mutants were generated and tested for their ability to: (i) suppress GFP silencing in a leaf patch assay on *Nicotiana benthamiana*; and (ii) to elicit *Tsw*-mediated HR on *Capsicum annuum* (*Tsw*⁺). Therefore, the NSs sequences obtained from a TSWV resistance-inducing (RI) isolate (Vir127) and an RB isolate (Vir160), and referred to as NSs^{RI} and NSs^{RB}, respectively (de Ronde *et al.*, 2013), were used to generate mutants.

The previously identified affinity of NSs to long and small dsRNA implied an important role for RNA-binding domains in RSS activity (Schnettler *et al.*, 2010). For this reason, the first set of mutants was generated from NSs^{RI} in which amino acids from three predicted RNA-binding domains according to BindN (Wang and Brown, 2006), and matching potentially exposed domains predicted from hydrophobicity plots (Fig. 1), were substituted for alanines, as described in Experimental Procedures, resulting in mutants designated S48A, S48A/R51A, K53A/G57A, R211A and KKK452/K457A (Table 1).

A second set of mutants was generated from NSs^{RI} based on multiple sequence alignment of different *Tospovirus* NSs

sequences (Fig. S1, see Supporting Information). Amino acid residues within conserved domains were selected and substituted for alanines to test the importance of these domains for RSS and Avr activity. The resulting mutants were denoted S29A/Y30A, S74A/Q75A, Q113A/G114A, G160A/S161A, Y197A/S198A, N288A/S289A, N355A/N356A, P373A, L396A/S397A and S411A/Y412A (Table 1).

To identify the amino acid residue(s) within NSs^{RI} that are important for the appearance of RB strains, a third set of mutants was generated in which single amino acid residues within NSs^{RB160}, unique and different from the NSs^{RI} sequence (de Ronde *et al.*, 2013), were reverted into amino acids from the NSs^{RI} strains (Fig. 1). The constructs obtained, denoted P48S, T79I, T260V, D384N, N386S, D384N/N386S and P438S, were tested for a gain of RSS and/or Avr function. In addition, the corresponding amino acid residues within the NSs^{RI} sequence were substituted for alanines, resulting in mutants denoted S48A, I79A, V260A, N384A, S386A, N384A/S386A and S438A (Table 1). The latter were tested for a loss of RSS and/or Avr activity.

In addition to these three sets of mutants a fourth mutant was constructed in which a GW/WG motif in the N-terminal domain of NSs was substituted for alanines, and denoted W17A/G18A. The GW/WG motif has recently been reported to be involved in the interaction of several RSS proteins with AGO1, the slicer component from RISC (Giner *et al.*, 2010).

All NSs mutants generated (Table 1) were confirmed by sequence analysis, and their translatability was verified by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis of *N. benthamiana* leaves agroinfiltrated with these constructs. Levels of (mutant) NSs expression differed depending on the transient expression vector used (pEAQ-HT versus pK2GW7), and was mostly good and comparable for all constructs when expressed from pEAQ-HT, but differed from pK2GW7 depending on the mutant involved (whether or not affected in RSS activity) [Fig. S2 (see Supporting Information) and following sections].

Essential amino acid sequences for Avr activity primarily map to the N-terminus

To test the ability of NSs^{RI} mutants and NSs^{RB160} revertants to trigger an HR, constructs were cloned into pEAQ-HT and subsequently transiently expressed via infiltration of *Agrobacterium tumefaciens* 1D1249 (containing helper plasmid pCH32) on resistant *C. annuum* plants (de Ronde *et al.*, 2013). Plants were monitored for the appearance of HR at 5 days post-agroinfiltration (dpa). The NSs mutants were expressed from vector pEAQ-HT to compensate (via the additional expression of the RSS P19) for the low expression levels in cases in which the NSs mutants were nonfunctional for RSS activity, and without triggering HR by P19 [Fig. S2 shows a set of selected NSs constructs analysed and

Fig. 1 Multiple sequence alignment of NSs^{RI}, NSs^{RB} and *Groundnut ringspot virus* (GRSV) NSs. A multiple sequence alignment of *Tomato spotted wilt virus* (TSWV) NSs^{RI} and TSWV NSs^{RB} from isolates 160 and 171 is shown and differences are highlighted. The NSs from a closely related but distinct *Tospovirus* GRSV is included. The predicted (three) RNA-binding domains (black bars), a WG/GW motif (grey bar) and a hydrophobicity plot (to identify potentially exposed amino acid domains) are indicated.

representative for the expression of all (mutant) NSs from pEAQ-HT] (de Ronde *et al.*, 2013). Although infiltration with pEAQ-HT-NSs^{RI} (control), but not with pEAQ-HT-N (nucleocapsid protein of TSWV) or pEAQ-HT-NSs^{RB160}, led to a clear HR after 5 days, quite a number of NSs^{RI} mutants lost their ability to trigger HR (Figs 2 and 3, Table 1). A further examination showed that all mutations in the N-terminal domain (amino acids 1–133) led to a loss of the ability to elicit HR, whereas only half of the other mutants in the remaining internal or C-terminal domain led to such loss. These results indicated the importance of the N-terminal domain of NSs function for Avr activity. Mutant N384A/S386A lost the ability to trigger HR, whereas the individual single mutants N384A and S386A were still functional. Furthermore, and interestingly, mutation of the WG motif also led to a loss of Avr activity, indicating its putative biological relevance. From all NSs^{RB160} single-amino-acid reversion mutants generated, none regained the ability to trigger HR. As control, all mutant NSs constructs were additionally tested on susceptible *Capsicum* plants, and none were shown to trigger an aspecific necrotic response (Fig. S3, see Supporting Information).

Essential sequences for RSS activity overlap with those for Avr activity

To test NSs mutants (NSs^{RI} alanine substitutions and NSs^{RB160} revertants) for their ability to suppress RNA silencing, constructs were cloned into pK2GW7 and, by means of *A. tumefaciens* LBA4404 (de Ronde *et al.*, 2013), co-infiltrated with a functional GFP construct in *N. benthamiana*. Plasmid pK2GW7 does not co-express P19 or any other viral RSS to compensate for low expression levels, and NSs mutated in domains that would compromise RSS activity would thus not be able to prevent its own silencing. As a consequence, low expression levels from pK2GW7 as such would already indicate a (partial) loss of RSS activity, as observed and demonstrated previously for the *Rice hoja blanca tenuivirus* NS3 RSS (Hemmes *et al.*, 2009). As expected, Western immunoblot analysis of mutant NSs indeed showed a large variation in protein expression, indicating a loss of RSS activity [Fig. S2 shows a set of selected NSs constructs analysed and representative for the expression of all (mutant) NSs from pK2GW7]. At 5 dpa, levels of GFP expression were monitored visually by UV light, and

#	Mutant	Mutant target	HR induction	RSS activity
1	NSs ^{RI} -W17A/G18A	Putative AGO1 interaction domain	–	–
2	NSs ^{RI} -S29A/Y30A	Conserved NSs domain	–	–
3	NSs ^{RI} -S48A	Predicted RNA-binding domain	–	+/-
4	NSs ^{RI} -S48A/R51A	Predicted RNA-binding domain	–	–
5	NSs ^{RI} -K53A/G57A	Predicted RNA-binding domain	–	+
6	NSs ^{RI} -S74A/Q75A	Conserved NSs domain	–	+
7	NSs ^{RI} -I79A	RB mutation	–	–
8	NSs ^{RI} -Q113A/G114A	Conserved NSs domain	–	–
9	NSs ^{RI} -G160A/S161A	Conserved NSs domain	+	+
10	NSs ^{RI} -Y197A/S198A	Conserved NSs domain	–	+/-
11	NSs ^{RI} -R211A	Predicted RNA-binding domain	+	–
12	NSs ^{RI} -V260A	RB mutation	–	+
13	NSs ^{RI} -N288A/S289A	Conserved NSs domain	–	+
14	NSs ^{RI} -N355A/N356A	Conserved NSs domain	–	–
15	NSs ^{RI} -P373A	RB mutation	+	+
16	NSs ^{RI} -N384A	RB mutation	+	+
17	NSs ^{RI} -S386A	RB mutation	+	+
18	NSs ^{RI} -N384A/S386A	RB mutation	–	+
19	NSs ^{RI} -L396A/S397A	Conserved NSs domain	–	+/-
20	NSs ^{RI} -S411A/Y412A	Conserved NSs domain	–	+
21	NSs ^{RI} -S438A	RB mutation	+	+
22	NSs ^{RI} -KKK452AAA/K457A	Predicted RNA-binding domain	–	+
23	NSs ^{RB160} -P48S	Reversion mutation	–	–
24	NSs ^{RB160} -T79I	Reversion mutation	–	–
25	NSs ^{RB160} -T260V	Reversion mutation	–	–
26	NSs ^{RB160} -D384N	Reversion mutation	–	–
27	NSs ^{RB160} -N386S	Reversion mutation	–	–
28	NSs ^{RB160} -D384N/N386S	Reversion mutation	–	–
29	NSs ^{RB160} -P438S	Reversion mutation	–	–

AGO1, Argonaute 1; HR, hypersensitive response; RB, resistance breaking; RSS, RNA silencing suppressor.

fluorescence units were quantified by spectrometry. Although agroinfiltration of GFP only showed clear levels of silencing, which were suppressed in the additional presence of NSs^{RI}, a variety of GFP silencing suppression levels were observed when the NSs^{RI} mutants were co-infiltrated (Fig. 2, Table 1). Similar to the results from the mutant screen on HR induction/elicitation, loss of RSS activity was mainly found with mutations that resided in the N-terminal domain, whereas fewer mutations in the remaining part of NSs had such an effect. Mutations made in the first two predicted RNA-binding domains (amino acids 48 and 211) showed a negative effect on RSS function, whereas those made in the third predicted RNA-binding domain (amino acid 452) did not show any effect on RSS functioning. Some NSs^{RI} mutants, i.e. S48A, Y197A/S198A and L396A/S397A, only partially lost their RSS activity, whereas the double mutant S48A/R51A completely lost its RSS activity. Mutation of the WG motif, in addition to Avr activity, also

abrogated the RSS activity of NSs. Furthermore, as with the HR induction assay, none of the single NSs^{RB160} reversion mutants regained RSS activity.

Elicitation of HR and RSS activity of NSs chimeras and additional mutants

The results from the NSs^{RB160} revertants indicated that a single nucleotide reversion was apparently not sufficient to restore either one or both of the RSS and Avr activities. Interestingly, a further examination of the N-terminal amino acid sequences of NSs^{RB} from the 160 and 171 RB strains in comparison with NSs^{RI} (Fig. 1) showed that NSs^{RB171} only deviated from NSs^{RI} at one position, namely amino acid 79 (T79). Considering the importance of the N-terminal domain for both RSS and Avr activity, we next analysed whether T79 represented one of the key amino acid residues for

Fig. 2 Hypersensitive response (HR) and RNA silencing suppressor (RSS) activity of NSs mutants. The NSs mutants listed in Table 1 were tested for their avirulence (Avr) activity by the triggering of HR (necrosis) on *Tsw+* *Capsicum* plants. The presence of RSS activity was analysed in a green fluorescent protein (GFP) silencing leaf patch assay on *Nicotiana benthamiana*. (A) Visual monitoring of the Avr activity from all the mutants tested and ordered from top left to bottom right according to the mutations positioned in NSs from the N-terminal to C-terminal end. (B) Suppression of GFP silencing by the NSs mutants used and presented in the same order as in (A). The presence of Avr activity and (full/intermediate/loss) of RSS activity are summarized and presented in Table 1. (C) The GFP units obtained in the RSS assay were quantified by fluorescence spectrometry. Measurements of leaf discs (diameter, 1 cm) of each infiltration were repeated at least three times and the average amount is shown with the standard deviations as error bars.

Fig. 3 Graphical overview of the NSs mutant analysis. The outcome of avirulence (Avr) and RNA silencing suppressor (RSS) functionality from the NSs^{RI} mutants is depicted in a schematic drawing including the predicted secondary NSs structure (α -helix in white boxes and β -sheets in dark grey boxes); the hydrophobicity plot is shown at the top of the figure and predicted RNA-binding domains are shown as horizontal black bars.

the generation of the TSWV RB phenotype/strains. To this end, T79 in NSs^{RB171} was reverted into isoleucine (T79I) and, after cloning into the appropriate vectors, subsequently tested for HR induction and RSS activity. Interestingly, the NSs^{RB171}-T79I mutant gained RSS activity again (Fig. 5), but not the ability to elicit HR. To analyse whether the reversion of two amino acids in the N-terminal domain of NSs^{RB160}, and deviating from NSs^{RI}, restored full functionality of RSS and Avr activity, the double mutant P48S/T79I was generated. In contrast with the results with the single reversion mutant NSs^{RB171}-T79I, which shared a similar N-terminal domain to the double reversion mutant NSs^{RB160}-P48S/T79I, the latter mutant showed a gain of function for both activities (Fig. 5).

To further substantiate the importance of the N-terminus of NSs in both activities, additional NSs chimeras were made. To this end, the first 133 amino acids (N-terminal domain) or the remaining part of NSs (referred to as the C-terminal domain) were swapped between NSs^{RI} and NSs^{RB160} or NSs^{RB171}, leading to the construction of four NSs chimeras (Fig. 4). All NSs chimera constructs were tested for their ability to suppress GFP silencing in a leaf patch assay and to elicit a *Tsw* gene-based HR. The results showed that NSs chimeras containing the N-terminal domain of NSs^{RI} and the remaining C-terminal part of NSs^{RB} (160 or 171) again showed RSS and Avr functionality (Fig. 5). Conversely, the NSs chimera containing the N-terminal domain of NSs^{RB} and the remaining C-terminal part of NSs^{RI} showed no Avr activity. Interestingly, residual/low levels of RSS activity were observed with the chimera containing the N-terminal domain of NSs^{RB171}, but not with the corresponding domain from NSs^{RB160}. As only few mutations in the C-terminus of NSs^{RI} led to a loss of either RSS or Avr activity, a C-terminal deletion mutant of NSs^{RI} was made in which the last 97 amino acids of the NSs^{RI} coding region were deleted and two stop codons were introduced. Analysis of this construct, referred to as NSs^{RI}- Δ C, showed that both RSS and Avr activity were abolished, indicating that the C-terminus of NSs is required to retain a functional protein.

Inability to transfer Avr activity to a closely related, but virulent, *Tospovirus* species

The observation that the N-terminus (133 amino acids) of NSs^{RI} seemed to be most essential for RSS and Avr activity tempted us to analyse whether Avr could be transferred from a TSWV^{RI} strain to *Groundnut ringspot virus* (GRSV). The latter represents a distinct *Tospovirus* species which is not able to elicit *Tsw*-mediated HR, but whose NSs shares high sequence similarity to TSWV NSs (89%). To this end, and in analogy with the approach described above, the first 133 amino acids of GRSV NSs were exchanged with the corresponding part of NSs^{RI} of TSWV, and vice versa. All NSs chimeras generated (Table 2) were confirmed by sequence analysis, and verified for their translatability as described previously (data not shown). During a GFP silencing assay on *N. benthamiana*, the chimera with the N-terminal domain of TSWV NSs showed the ability to suppress RNA silencing to the same level as the complete NSs^{RI}, but the chimera carrying the N-terminal domain of GRSV NSs partially lost RSS activity (intermediate RSS activity). However, when both chimeras were tested for Avr, neither chimera was able to elicit *Tsw*-mediated HR.

DISCUSSION

In this study, a mutant screen was performed on the NSs protein of TSWV to identify and map essential domains required for RSS and Avr activity, and to investigate their potential tight functional linkage. The results strongly indicated the importance of the N-terminal domain of NSs for both functions, as mutations introduced in the first 133 amino acids of NSs^{RI} most often led to a functional loss of both RSS and Avr activity, whereas this was observed only occasionally and to a lesser extent with mutations in the remaining C-terminal part of the protein. A few mutants were affected only in RSS or Avr activity. This observation dismissed the idea of a tight functional linkage. This was further supported by data from another study (Margaria *et al.*, 2007) in

Fig. 4 Schematic presentation of additional NSs chimeras and the NSs^{RI}-ΔC mutant construct. (A) Presentation of four NSs chimera in which the first 133 amino acids between NSs^{RI} and NSs^{RB160} and NSs^{RB171} were swapped. (B) Presentation of NSs-ΔC, an NSs^{RI} deletion mutant from which the last 95 amino acids on the C-terminal end were deleted. (C) Presentation of the NSs chimera made from *Groundnut ringspot virus* (GRSV) NSs and *Tomato spotted wilt virus* (TSWV) NSs^{RI}, similar to the constructs in (A), in which the first 133 amino acids between NSs^{GRSV} and NSs^{RI} from TSWV have been swapped.

Fig. 5 Hypersensitive response (HR) induction and RNA silencing suppressor (RSS) activity of the NSs domain swapping chimera. Leaves in the top row show the results of the HR assay of the NSs chimera tested, similar to (A) in Fig. 2. Leaves in the bottom row show the results of the green fluorescent protein (GFP) silencing suppression assay of the mutants as shown in (B) of Fig. 2. The presence of avirulence (Avr) activity and (full/intermediate/loss) of RSS activity of the mutants is summarized and presented in Table 2.

which the NSs protein of some RB isolates, although only partially characterized, still seemed to exhibit RSS activity.

The importance of the N-terminal domain for both functions was further strengthened by additional domain swaps between NSs^{RI} and NSs^{RB}, in which the RSS/Avr activity of the NSs^{RI}-RB chimera could be restored when provided with the N-terminal part of NSs^{RI}. In addition, only mutants of NSs^{RB160-171} containing a reversion in the N-terminal domain, but not in the C-terminal part, were (partially) restored in RSS and/or Avr activity. Although the mutant screen indicated a lower importance of the C-terminal domain of TSWV NSs for both functions, deletion of this part (mutant NSs^{RI}-ΔC) rendered NSs nonfunctional. This suggests that the C-terminal domain probably plays a structural role, rather than possessing domains required for the tested functions. Furthermore, a chimeric GRSV NSs containing the N-terminal domain of TSWV NSs^{RI} could not provide Avr functionality, but retained its

RSS activity, which strengthens the requirement for the C-terminal domain of NSs for correct functioning, probably in structural folding. Mutation of the WG motif in the N-terminus of NSs and concomitant loss of RSS and Avr activity pointed to its potential biological relevance, probably for interaction with AGO1, as demonstrated previously with *Sweet potato mild mottle virus* P1 protein (Giner *et al.*, 2010). From all amino acid mutations in the NSs protein analysed, the residue at position 79 also seems to play a key role, as mutant NSs^{RI}-I79A lost both activities, whereas reversion mutants of the resistance breakers, i.e. NSs^{RB171}-T79I and NSs^{RB160}-P48S/T79I, both gained RSS ability and the latter, in addition, Avr activity. Clearly, amino acid residue 79 is directly involved in RSS activity, but also affects Avr activity.

Of the known viral Avr proteins, only a small number of cases have been reported in which the viral Avr also shows RSS activity. These are limited to *Tomato bushy stunt virus* (TBSV) P19, *Tomato*

Table 2 Additional NSs mutants and chimeras made and tested with regard to their functionality: –, no or loss of activity; +, activity (gained); +/-, partial activity.

#	Mutant	Mutant target	HR induction	RSS activity
30	NSs ^{RB171} -T79I	Reversion mutation	–	+
31	NSs ^{RB160} -P48S/T79I	Reversion mutation	+	+
32	NSs ^{RI} -N-term/NSs ^{RB160} -C-term	Replacement N-terminus	+	+
33	NSs ^{RI} -N-term/NSs ^{RB171} -C-term	Replacement N-terminus	+	+
34	NSs ^{RB160} -N-term/NSs ^{RI} -C-term	Replacement N-terminus	–	–
35	NSs ^{RB171} -N-term/NSs ^{RI} -C-term	Replacement N-terminus	–	+/-
36	NSs ^{RI} -ΔC-term	C-terminal deletion	–	–
37	NSs ^{TSWV} -N-term/NSs ^{GRSV} -C-term	Replacement N-terminus	–	+
38	NSs ^{GRSV} -N-term/NSs ^{TSWV} -C-term	Replacement N-terminus	–	+/-

HR, hypersensitive response; RSS, RNA silencing suppressor.

aspermy virus (TAV) 2b, *Potato virus X* (PVX) 25K, *Turnip crinkle virus* (TCV) coat protein (CP) and *Potato virus Y* (PVY) helper component proteinase (Hc-Pro) (Angel *et al.*, 2011; Li *et al.*, 1999; Malcuit *et al.*, 1999; Oh *et al.*, 1995; Scholthof *et al.*, 1995; Tian and Valkonen, 2012). Although, for most of these cases, the corresponding *R* genes have not yet been cloned (TBSV, PVX, TAV and PVY), they are single dominant and therefore probably of the NBS-LRR type. The most well-known viral Avr and RSS *R*-gene model is that from TCV. Like TSWV NSs, the TCV CP binds dsRNA and prevents systemic silencing (Qu *et al.*, 2003). The TCV CP is the Avr determinant of the *HRT* *R* gene in *Arabidopsis thaliana*, where *HRT* is likely to be an NBS-LRR-type *R* gene (Ren *et al.*, 2000). For TCV CP, mutants were made that lost the ability to induce HR, but remained RSS active, suggesting that the suppressor and resistance elicitor functions are not tightly linked, most probably because these reside in different domains, which tempted the authors to propose that TCV CP interferes with multiple host basal defence pathways (Choi *et al.*, 2004; Jeong *et al.*, 2008). Similar findings were obtained for TBSV, where P19 mutants lost siRNA-binding properties, but were still able to trigger an effective resistance response (HR) in *N. tabacum* (Hsieh *et al.*, 2009). Recently, the TAV 2b crystal structure has been elucidated, and two separate studies have shown the importance of the same arginine residue at position 28 from TAV 2b in the binding of siRNAs, and thus for RSS activity (Chen *et al.*, 2008) and Avr activity (Li *et al.*, 1999), indicating that RSS and Avr activity in the case of TAV 2b are tightly linked. Most of these studies support the idea that triggering of the corresponding *R* genes does not require a functional RSS aspect of the Avr determinant, as in the case of TSWV NSs, but rather a (minimal secondary) structural feature that is shared by both, and, in the case of TSWV NSs, could be the N-terminal domain. Support for this also comes from another recent study on

Cucumber mosaic virus (CMV), where the replicase function of 2a could be uncoupled from its ability to trigger an HR on cowpea leaves (unknown *R* gene) (Hu *et al.*, 2012). However, for some viruses, the resolution of this issue might be hampered by the difficulty to separate functions in—multifunctional—viral proteins and, as a consequence, only functional viral RSS proteins may be recognized by *R* proteins, which could explain the observations made on TAV 2b.

Recently, TSWV NSs has been shown to bind small RNAs (microRNA/siRNA) and long dsRNA, and this characteristic has been proposed as the mode by which this protein interferes in (suppressing) RNA silencing (Schnettler *et al.*, 2010). Alanine substitutions in three predicted RNA-binding domains showed that only two of these led to a loss of RSS activity. The third, at around amino acid 452, in which even four lysines were substituted, did not lead to a loss of RSS activity, and suggested that this sequence was not required. In contrast, a similar sequence with predicted RNA-binding capacity has been shown previously to be involved in small RNA binding by the Tenuivirus *Rice hoja blanca virus* NS3, and its mutation leads to a loss of RSS activity (Hemmes *et al.*, 2009). Whether the predicted RNA-binding domains are genuinely involved in the binding of small and/or large dsRNA has not been resolved so far. In the light of this, it is important to point out that attempts have been made to perform electrophoretic mobility shift assays (EMSAs) using transiently expressed NSs, but mostly only weak shifts were observed when using NSs^{RI}, whereas no shift was observed using NSs^{RB} (de Ronde *et al.*, 2013). Considering that NSs^{RI} was easily detectable on Western immunoblots, but the amount of NSs^{RB}, and of most mutant NSs proteins, was at least 10-fold lower, indicates that the absence of a shift could also be a result of low expression levels, and the outcome of EMSA would thus not be conclusive.

The same predicted RNA-binding domains are also present in other closely related *Tospovirus* sequences (Fig. S1), and indicate that these could be a conserved and shared feature throughout the genus. Among all the NSs mutants analysed, one (R211A) lost RSS activity, but was still able to trigger HR. Considering that the R211A mutant expressed from pEAQ-HT is still able to trigger HR, whereas its expression is 10-fold lower when compared with NSs^{RI} from pEAQ-HT, indicates that the induction of HR apparently requires only small amounts of the Avr protein.

The crystal structure of the TSWV NSs protein and other *Tospovirus* NSs proteins has not been elucidated, and therefore the three-dimensional structure of the NSs protein is still unknown. This clearly hampers the predictability of the outcome of the mutations in the NSs protein sequence, and we cannot rule out that a loss of function as a result of some mutations may be caused by an effect on the structure of the protein, rather than the disruption of a functional domain. The elucidation of the structure of NSs might also provide clues as to how and why mutation of the WG motif affects the Avr function of NSs.

Recently, a publication has appeared on the biochemical function of NSs from *Groundnut bud necrosis virus* (GBNV), a distinct *Tospovirus* (Lokesh *et al.*, 2010). It was shown that GBNV NSs contains RNA-stimulated NTPase activity and dATPase activity, which was supported by the presence of two Walker motifs (A and B) in the NSs amino acid sequence. In addition, the NSs showed 5' RNA/DNA phosphatase activity. Although the Walker domains cannot be detected in the TSWV NSs sequence, the presence of similar biochemical functions for this protein cannot be excluded nor can their involvement in the RSS/Avr activity of the NSs protein. Unfortunately, the NSs mutants prepared in the GBNV study were not tested for their biological relevance, e.g. RNAi suppressor activity.

In another more recent study (Geerts-Dimitriadou *et al.*, 2012), TSWV NSs has been shown to enhance the translation of viral transcripts. It is clear from this that NSs represents a multifunctional protein, as observed with many other viral proteins (Díaz-Pendón and Ding, 2008; Hu *et al.*, 2012). Whether any of these functions overlap or somehow crosstalk/interplay with RSS/Avr activity remains to be analysed.

In conclusion, we have performed a first mutant screen of TSWV NSs which provides a first glimpse of the topology of NSs in relation to its role as an RSS and Avr determinant, and shows that the most essential sequences required for both functions map to the N-terminal part of the protein and closely overlap, although both functions can be separated. Although the mode of action of dominant *R* genes, to which *Tsw* classifies, still remains a matter of debate, one of the most commonly accepted models is the guard hypothesis (van der Biezen and Jones, 1998; Jones and Dangl, 2006). In this model, the *R*-gene product guards a certain host protein, and is able to perceive alterations of its 'guardee' target by interaction with the Avr determinant, which leads to an induction of HR. Whether AGO1 indeed interacts with NSs, and presents a candidate protein for the guardee, would be an obvious hypothesis to be tested in the near future.

EXPERIMENTAL PROCEDURES

Plant material

Two genotypes of *C. annuum* were used for the HR induction assay: HK0004, a TSWV-susceptible cultivar (*-Tsw*), and YF0009, a TSWV-resistant cultivar (*+Tsw*). In addition, *N. benthamiana* plants were used for the RNA silencing suppression assay. All plants were grown and maintained under glasshouse conditions (24 °C with a 16-h light/8-h dark regime).

Multiple sequence alignment

The sequences obtained were aligned using the CLUSTALW algorithm. Sequences of NSs^{RI}, NSs¹⁶⁰ and NSs¹⁷¹ have been described previously

(de Ronde *et al.*, 2013). Sequences of NSs derived from other tospoviruses were retrieved from GenBank: TSWV NSs, GenBank accession number D00645; *Zucchini lethal chlorosis virus* (ZLCV) NSs, accession number JN572104; GRSV NSs, accession number JN571117; *Impatiens necrotic spot virus* (INSV) NSs, accession number GU112504; *Chrysanthemum stem necrosis virus* (CSNV) NSs, accession number AB600873. Alignments were edited using the BioEdit program (Hall, 1999).

Polymerase chain reaction (PCR) mutagenesis and subcloning of constructs

NSs mutants were made by PCR mutagenesis, using primers to introduce the mutation on a Gateway-compatible entry vector pEntr11, carrying the NSs^{RI}, NSs^{RB160} or NSs^{RB171} sequence. Chimera mutants were made by amplifying fragments by PCR with introduced restriction sites for subsequent restriction and ligation into the appropriate backbone sequence. Plasmids obtained were sequence verified and subsequently cloned by Gateway cloning into their destination vectors pK2GW7 (Karimi *et al.*, 2002) for the GFP silencing suppression assay and pEAQ-HT (Sainsbury *et al.*, 2009) for the Avr assay. The binary vectors obtained were transformed into *Agrobacterium* 1D1249 + pCH32 (pEAQ-HT) and LBA4404 (pK2GW7) (Hamilton *et al.*, 1996; Ooms *et al.*, 1982; Wroblewski *et al.*, 2005).

Agrobacterium transient transformation assay (ATTA)

ATTA was performed according to Bucher *et al.* (2003), with slight modifications. In brief, agrobacteria were grown overnight at 28 °C in LB3 medium containing appropriate antibiotic selection pressure. From this culture, 600 µL were freshly inoculated into 3 mL of induction medium and grown overnight. Plants were watered in excess 1 h before infiltration, using a needleless syringe, was performed. Strain *A. tumefaciens* 1D1249 with helper plasmid pCH32 was grown under 1.25 µg/mL tetracycline selection pressure, whereas *A. tumefaciens* LBA4044 was grown under 20 µg/mL rifampicin selection pressure.

Serological detection of NSs proteins

The expression of the different mutants of the TSWV NSs protein was analysed by SDS-PAGE and Western immunoblot analysis using polyclonal antisera against TSWV NSs, as described previously (Kormelink *et al.*, 1991; de Ronde *et al.*, 2013).

HR induction assay and GFP silencing suppression assay

For HR induction, ATTAs (see above) were performed of the pEAQ-NSs mutant constructs on resistant (*+Tsw*) and susceptible (*-Tsw*) *Capsicum* plants, and the plants were scored for the presence or absence of HR. NSs^{RI} was included as a positive control, and NSs^{RB160} and the nucleocapsid (N) protein of TSWV were included as negative controls (de Ronde *et al.*, 2013). The GFP silencing assay was performed by agroinfiltration of *N. benthamiana* leaves with a functional GFP construct (Tsien, 1998) mixed with one of the NSs constructs, as described above in the ATTA

section, using a final optical density at 600 nm ($OD_{600\text{ nm}}$) of 0.25 per construct. A construct expressing maltose binding protein (MBP) was used as a negative control (Schnettler *et al.*, 2010). Infiltrated leaves were monitored for GFP expression at 5 dpa using a hand-held UV lamp. For the quantification of GFP fluorescence, leaf discs with a diameter of 1 cm were taken from the infiltrated leaf area and the number of fluorescent units was measured using a Fluorstar Optima (BMG Labtech, Ortenberg, Germany).

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REFERENCES

- Angel, C.A., Hsieh, Y.-C. and Schoelz, J.E. (2011) Comparative analysis of the capacity of Tombusvirus P22 and P19 proteins to function as avirulence determinants in *Nicotiana* species. *Mol. Plant-Microbe Interact.* **24**, 91–99.
- van der Biezen, E.A. and Jones, J.D.G. (1998) The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. *Curr. Biol.* **8**, R226–R227.
- Bucher, E., Sijen, T., De Haan, P., Goldbach, R. and Prins, M. (2003) Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *J. Virol.* **77**, 1329–1336.
- Chen, H.Y., Yang, J., Lin, C. and Yuan, Y.A. (2008) Structural basis for RNA-silencing suppression by *Tomato aspermy virus* protein 2b. *EMBO Rep.* **9**, 754–760.
- Choi, C.W., Qu, F., Ren, T., Ye, X. and Morris, T.J. (2004) RNA silencing-suppressor function of *Turnip crinkle virus* coat protein cannot be attributed to its interaction with the *Arabidopsis* protein TIP. *J. Gen. Virol.* **85**, 3415–3420.
- Díaz-Pendón, J.A. and Ding, S.-W. (2008) Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annu. Rev. Phytopathol.* **46**, 303–326.
- Ding, S.-W. and Voinnet, O. (2007) Antiviral immunity directed by small RNAs. *Cell*, **130**, 413–426.
- Geerts-Dimitriadou, C., Lu, Y.-Y., Geertsema, C., Goldbach, R. and Kormelink, R. (2012) Analysis of the *Tomato spotted wilt virus* ambisense S RNA-encoded hairpin structure in translation. *PLoS ONE*, **7**, e31013.
- Giner, A., Lakatos, L., García-Chapa, M., López-Moya, J.J. and Burguán, J. (2010) Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. *PLoS Pathog.* **6**, e1000996.
- Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98.
- Hamilton, C.M., Frary, A., Lewis, C. and Tanksley, S.D. (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proc. Natl. Acad. Sci. USA*, **93**, 9975–9979.
- Hemmes, H., Kaaij, L., Lohuis, D., Prins, M., Goldbach, R. and Schnettler, E. (2009) Binding of small interfering RNA molecules is crucial for RNA interference suppressor activity of *Rice hoja blanca virus* NS3 in plants. *J. Gen. Virol.* **90**, 1762–1766.
- Hsieh, Y.C., Omarov, R.T. and Scholthof, H.B. (2009) Diverse and newly recognized effects associated with short interfering RNA binding site modifications on the *Tomato bushy stunt virus* p19 silencing suppressor. *J. Virol.* **83**, 2188–2200.
- Hu, Z., Zhang, T., Yao, M., Feng, Z., Miriam, K., Wu, J., Zhou, X. and Tao, X. (2012) The 2a protein of *Cucumber mosaic virus* induces a hypersensitive response in cowpea independently of its replicase activity. *Virus Res.* **170**, 169–173.
- Incarbone, M. and Dunoyer, P. (2013) RNA silencing and its suppression: novel insights from in planta analyses. *Trends Plant Sci.* **18**, 382–392.
- Jeong, R.-D., Chandra-Shekara, A.C., Kachroo, A., Klessig, D.F. and Kachroo, P. (2008) HRT-mediated hypersensitive response and resistance to *Turnip crinkle virus* in *Arabidopsis* does not require the function of TIP, the presumed guard protein. *Mol. Plant-Microbe Interact.* **21**, 1316–1324.
- Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.
- Karimi, M., Inze, D. and Depicker, A. (2002) Gateway(TM) vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* **7**, 193–195.
- Kormelink, R., Kitajima, E.W., Dehaan, P., Zuidema, D., Peters, D. and Goldbach, R. (1991) The nonstructural protein (NSs) encoded by the ambisense S-RNA segment of *Tomato spotted wilt virus* is associated with fibrous structures in infected plant cells. *Virology*, **181**, 459–468.
- Kormelink, R., Garcia, M.L., Goodin, M., Sasaya, T. and Haenni, A.-L. (2011) Negative-strand RNA viruses: the plant-infecting counterparts. *Virus Res.* **162**, 184–202.
- Li, H.W., Lucy, A.P., Guo, H.S., Li, W.X., Ji, L.H., Wong, S.M. and Ding, S.W. (1999) Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. *EMBO J.* **18**, 2683–2691.
- Lokesh, B., Rashmi, P.R., Amruta, B.S., Srisathyanarayanan, D., Murthy, M.R.N. and Savithri, H.S. (2010) NSs encoded by *Groundnut bud necrosis virus* is a bifunctional enzyme. *PLoS ONE*, **5**, e9757.
- Lovato, F.A., Inoue-Nagata, A.K., Nagata, T., de Avila, A.C., Pereira, L.A. and Resende, R.O. (2008) The N protein of *Tomato spotted wilt virus* (TSWV) is associated with the induction of programmed cell death (PCD) in *Capsicum chinense* plants, a hypersensitive host to TSWV infection. *Virus Res.* **137**, 245–252.
- Malcuit, I., Marano, M.R., Kavanagh, T.A., De Jong, W., Forsyth, A. and Baulcombe, D.C. (1999) The 25-kDa movement protein of PVX elicits Nb-mediated hypersensitive cell death in potato. *Mol. Plant-Microbe Interact.* **12**, 536–543.
- Margaria, P., Ciuffo, M., Pacifico, D. and Turina, M. (2007) Evidence that the nonstructural protein of *Tomato spotted wilt virus* is the avirulence determinant in the interaction with resistant pepper carrying the *Tsw* gene. *Mol. Plant-Microbe Interact.* **20**, 547–558.
- Moffett, P. (2009) Mechanisms of recognition in dominant *R* gene mediated resistance. *Adv. Virus Res.* **75**, 1–33.
- Oh, J.W., Kong, Q., Song, C., Carpenter, C.D. and Simon, A.E. (1995) Open reading frames of *Turnip crinkle virus* involved in satellite symptom expression and incompatibility with *Arabidopsis thaliana* ecotype Dijon. *Mol. Plant-Microbe Interact.* **8**, 979–987.
- Ooms, G., Hooykaas, P.J.J., Van Veen, R.J.M., Van Beelen, P., Regensburg-Tuink, J.G.G. and Schilperoort, R.A. (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. *Plasmid*, **7**, 15–29.
- Qu, F., Ren, T. and Morris, T.J. (2003) The coat protein of *Turnip crinkle virus* suppresses posttranscriptional gene silencing at an early initiation step. *J. Virol.* **77**, 511–522.
- Ren, T., Qu, F. and Morris, T.J. (2000) HRT gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to *Turnip crinkle virus*. *Plant Cell*, **12**, 1917–1926.
- de Ronde, D., Butterbach, P., Lohuis, D., Hedil, M., van Lent, J.W.M. and Kormelink, R. (2013) *Tsw* gene-based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*. *Mol. Plant Pathol.* **14**, 405–415.
- Sainsbury, F., Thuenemann, E.C. and Lomonosoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol. J.* **7**, 682–693.
- Schnettler, E., Hemmes, H., Huismann, R., Goldbach, R., Prins, M. and Kormelink, R. (2010) Diverging affinity of *Tospovirus* RNA silencing suppressor proteins, NSs, for various RNA duplex molecules. *J. Virol.* **84**, 11 542–11 554.
- Scholthof, H.B., Scholthof, K.B. and Jackson, A.O. (1995) Identification of *Tomato bushy stunt virus* host-specific symptom determinants by expression of individual genes from a *Potato virus X* vector. *Plant Cell*, **7**, 1157–1172.
- Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S. and Okuno, T. (2002) Identification of a novel RNA silencing suppressor, NSs protein of *Tomato spotted wilt virus*. *FEBS Lett.* **532**, 75–79.
- Tian, Y.-P. and Valkonen, J.P.T. (2012) Genetic determinants of *Potato virus Y* required to overcome or trigger hypersensitive resistance to PVY strain group O controlled by the gene *Ny* in Potato. *Mol. Plant-Microbe Interact.* **26**, 297–305.
- Tsien, R.Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544.
- Wang, L. and Brown, S.J. (2006) BindN: a web-based tool for efficient prediction of DNA and RNA binding sites in amino acid sequences. *Nucleic Acids Res.* **34**, W243–W248.

- Wroblewski, T., Tomczak, A. and Micheltore, R. (2005) Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnol. J.* **3**, 259–273.
- Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J. and Chua, N.H. (2006) *Cucumber mosaic virus* encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes Dev.* **20**, 3255–3268.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Multiple sequence alignment of *Tospovirus* NSs proteins. The NSs amino acid sequences from different tospoviruses, belonging to the American clade, were aligned to identify conserved and diverse regions within the NSs protein. Conserved amino acids highlighted in black were selected for alanine substitutions. Black horizontal bars represent predicted RNA-binding domains. TSWV, *Tomato spotted wilt virus*; GRSV, *Groundnut ringspot virus*; CSNV, *Chrysanthemum stem necrosis virus*; ZLCV, *Zucchini lethal chlorosis virus*; INSV, *Impatiens necrotic spot virus*.

Fig. S2 Western blot analysis of chimeric NSs expressed from pEAQ-HT and pK2GW7. NSs chimera constructs made between NSs^{RI} and NSs^{RB160/171} were expressed in *Nicotiana benthamiana*, and leaves were subsequently prepared and analysed by Western blot analysis as described previously (de Ronde *et al.*, 2013), using α NSs as antiserum. Constructs tested were expressed from the pEAQ-HT vector (A) and from pK2GW7 (B). The chimera NSs^{RB160}-N-term/NSs^{RI}-C-term shown in (B) is RNA silencing suppressor (RSS) negative, whereas NSs^{RB171}-N-term/NSs^{RI}-C-term is partially RSS active (as shown in Fig. 5 and Table 2).

Fig. S3 Agroinfiltration of NSs mutants on susceptible *Capsicum* plants. All NSs mutants generated in this study (listed in Tables 1 and 2) were, as a control, agroinfiltrated in susceptible *Capsicum* plants to analyse their phenotype and to confirm the induction of the *Tomato spotted wilt* (*Tsw*)-mediated hypersensitive response (HR) in *Tsw*+ *Capsicum* plants (Figs 2 and 5). Agroinfiltrated leaves are presented from all mutants tested and ordered from top left to bottom right according to the mutations positioned in NSs from the N-terminal to C-terminal end, followed by the reversion and chimera/deletion mutants.