Tsw* gene-based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus

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SUMMARY

As a result of contradictory reports, the avirulence (Avr) determinant that triggers *Tsw* gene-based resistance in *Capsicum annuum* against the *Tomato spotted wilt virus* (TSWV) is still unresolved. Here, the *N* and *NSs* genes of resistance-inducing (RI) and resistance-breaking (RB) isolates were cloned and transiently expressed in resistant *Capsicum* plants to determine the identity of the Avr protein. It was shown that the *NSs*^{RI} protein triggered a hypersensitive response (HR) in *Tsw*-containing *Capsicum* plants, but not in susceptible *Capsicum*, whereas no HR was discerned after expression of the *N*^{RI/RB} protein, or when *NSs*^{RB} was expressed. Although *NSs*^{RI} was able to suppress the silencing of a functional green fluorescence protein (GFP) construct during *Agrobacterium tumefaciens* transient assays on *Nicotiana benthamiana*, *NSs*^{RB} had lost this capacity. The observation that RB isolates suppressed local GFP silencing during an infection indicated a recovery of RNA silencing suppressor activity for the *NSs* protein or the presence of another RNA interference (RNAi) suppressor. The role of *NSs* as RNA silencing suppressor and Avr determinant is discussed in the light of a putative interplay between RNAi and the natural *Tsw* resistance gene.

INTRODUCTION

The zigzag model (Jones and Dangl, 2006) is commonly accepted to illustrate the arms race between the plant immune system and plant pathogens. Within this model, the first line of defence is triggered by so-called microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively). These molecules are recognized by pattern recognition receptors (PRRs) and lead to the (slow) onset of PAMP-triggered immunity (PTI) (Chisholm *et al.*, 2006). Well-known PAMPs are bacterial flagellin and fungal chitin (Nicaise *et al.*, 2009). Pathogens encode virulence factors (effectors) that interfere with PTI, and thereby enable them to achieve a successful colonization/infection. In a third phase, these same effectors are specifically recognized by a second branch of the plant immune system that involves protein products from resistance (*R*) genes, and is called effector-triggered immunity (ETI). This recogni-

tion generally leads to a rapid hypersensitive response (HR), and involves a programmed cell death (PCD) at the infection site.

Although plant viruses are obligate parasites and replicate intracellularly, they too are subject to PTI. RNA silencing can be regarded as a PTI mechanism which enables plants and insects to clear viral infections (Ding and Voinnet, 2007). In this case, viral double-stranded RNA (dsRNA) molecules, either from replicative intermediates or folding structures, act as PAMPs, and their recognition leads to the induction of RNA silencing, also referred to as RNA interference (RNAi) (Ding and Voinnet, 2007). This defence mechanism involves dsRNA cleavage by an RNaseIII-like enzyme, called dicer-like (DCL), into small short interfering RNA (siRNA) duplex molecules. One strand of this duplex molecule, the so-called guide strand, is uploaded into an RNA-induced silencing complex (RISC), which then starts to survey and sense target (viral) RNA molecules with complementarity to the guide strand, leading to their degradation (Ding and Voinnet, 2007). Plant viruses have evolved different ways to counteract this antiviral defence mechanism. One of the most commonly used strategies is to encode RNA silencing suppressor (RSS) proteins (Díaz-Pendón and Ding, 2008). For many plant viruses, RSS proteins have been identified and have been shown to exert this function in a diverse manner, e.g. some RSSs sequester long or short siRNAs and thereby prevent their uploading into RISC or cleavage by DCL (Alvarado and Scholthof, 2009; Csorba *et al.*, 2009; Giner *et al.*, 2010; Lakatos *et al.*, 2006; Schnettler *et al.*, 2010; Vargason *et al.*, 2003). In other cases, the RSS protein prevents the maturation of the RISC or the cleavage of RNA target sequences (Ding and Voinnet, 2007).

As a result of the large economic impact of tospovirus diseases, ranking second on the list of the most important plant viruses worldwide (Scholthof *et al.*, 2011), the search for natural resistance genes in breeding programmes has received increasing interest. So far, only two single dominant resistance genes, *Sw5b* and *Tsw*, have been well described and are available for commercial resistance breeding against tospoviruses. *Sw5b* has been identified in *Solanum peruvianum* and provides high resistance levels to *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) (Boiteux & de B. Giordano, 1993; Stevens *et al.*, 1992). The *Tsw* gene (Black *et al.*, 1991; Boiteux, 1995; Jahn *et al.*, 2000) originates from *Capsicum chinense* 'PI' accessions and has been introgressed into *Capsicum annuum* cultivars. Resistance is displayed by an HR, as with *Sw5b*, which prevents systemic spread of the virus and eventually leads

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to leaf abscission (Boiteux and de Avila, 1994). The resistance only holds for isolates belonging to the species TSWV, and not to GRSV, TCSV or more distantly related tospoviruses (Boiteux and de Avila, 1994). As in the case of *Sw5b*, resistance-breaking (RB) variants of *Tsw* have been identified (Aramburu and Martí, 2003; Boiteux & de B. Giordano, 1993; Margaria *et al.*, 2004; Roggero *et al.*, 2002).

The viral gene product that triggers *Tsw* resistance has been mapped to the S RNA segment of TSWV (Jahn *et al.*, 2000), pointing towards either *N* or *NSs* as the *avirulence* (*Avr*) gene. In recent years, two reports have been published by Lovato *et al.* (2008) and Margaria *et al.* (2007), which revealed contradictory results on the identification of the *Avr* gene, leaving the identity of the *Avr* determinant unresolved.

Here, we have identified the *NSs* protein as the *Avr* determinant of *Tsw*-based resistance using a highly translatable transient expression vector construct.

RESULTS

Characterization of different TSWV isolates

To identify the *N* or *NSs* gene as the *Avr* determinant of *Tsw* resistance, several TSWV resistance-inducing (RI) and RB isolates were collected from different regions in Europe (Table 1). Prior to cloning and sequence analysis of the *N* and *NSs* genes, the TSWV isolates were verified for their phenotype on *Tsw*-resistant *Capsicum* plants by mechanical inoculation on *Capsicum* species (*C. annuum* + *Tsw*, *C. annuum* – *Tsw* and *C. chinense*) and on *Nicotiana benthamiana* as a positive control. Resistant *Capsicum*

plants inoculated with RI TSWV isolates Vir127 and Vir129 showed an HR 3–4 days post-inoculation (dpi) (Fig. 1) as necrotic lesions on the inoculated leaf. The small necrotic lesions appeared after 3 dpi (diameter, 1–2 mm) and expanded over time to large necrotic lesions (diameter, 4–5 mm) at ± 7 dpi, after which the whole leaf abscised. On these plants, no systemic symptoms (≥ 7 days) could be discerned, nor could the virus be detected by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) in these leaves. The RB isolates Vir160 and Vir171 did not induce HR on resistant *Capsicum* plants (Fig. 1), but produced clear systemic symptoms at 10–12 dpi and detectable levels of virus by DAS-ELISA. Susceptible *Capsicum* plants, challenged with all four isolates, showed typical TSWV symptoms, including local and systemic leaf chlorosis, vein yellowing, mottling and overall plant stunting at 10–12 dpi. The presence of virus was confirmed by DAS-ELISA. These results confirm the RI phenotype of isolates Vir127 and Vir129, and the RB phenotype of isolates Vir160 and Vir171.

Nucleotide sequence analysis of the *N* and *NSs* genes

To identify differences within the amino acid sequences of the *N* and *NSs* proteins of TSWV RI and RB isolates that could point towards the candidate *Avr* gene, these genes were cloned and their nucleotide sequences were determined. The amino acid sequences deduced from the *N* and *NSs* genes were used in a multiple sequence alignment to identify differences between the RI and RB isolates. To exclude sequence divergence as a result of polymorphism, the TSWV reference isolate Br01 was included in the alignments (de Avila *et al.*, 1990). In both alignments, several

TSWV isolate	Origin	Location	Collection date	Phenotype
Vir127	Romania	Unknown	1998	Resistance inducing
Vir129	The Netherlands	Wageningen University	2002	Resistance inducing
Vir160	Spain	Field Isolate Almeria	2006	Resistance breaking
Vir171	Spain	Field Isolate Almeria	2008	Resistance breaking

Table 1 The *Tomato spotted wilt virus* (TSWV) isolates used in this study.

Fig. 1 Local symptoms on resistant *Capsicum annuum* leaves at 5 days post-inoculation (dpi) with different *Tomato spotted wilt virus* (TSWV) isolates. TSWV Vir127, Vir129, Vir160 and Vir171 (from left to right, respectively) were mechanically inoculated on resistant *C. annuum* leaves. Vir127 and Vir129 induced a hypersensitive response (HR) (necrotic lesions), whereas Vir160 and Vir171 only induced chlorosis. Similar symptoms were observed with these isolates on *Capsicum chinense* (not shown). RB, resistance breaking; RI, resistance inducing.

Fig. 2 Alignment of N (a) and NSs (b) amino acid sequences of *Tomato spotted wilt virus* (TSWV) resistance-inducing (RI) and resistance-breaking (RB) isolates. The N and NSs protein sequences of the TSWV isolates Vir127, Vir129, Vir160 and Vir171 were aligned with the TSWV Br01 strain included as a reference. Highlighted in bold are the differences in amino acid residues between the RI and RB isolates. As both *N* and *NSs* genes show differences, no candidate *avirulence* (*Avr*) gene could be determined.

mutations were observed that were only present in the sequences of the N (Fig. 2a) and NSs (Fig. 2b) proteins of the RB isolates, and not in the RI isolates. Although some of these mutations were conserved (boxed black), differences were found in both *N* and *NSs* genes of the RB and RI isolates. These data did not provide support for one of the two genes as the candidate *Avr* gene.

Transient expression of the TSWV NSs protein triggers an HR in resistant *Capsicum* plants

Avr determinants of dominant resistance genes are commonly identified by the transient expression of candidate genes and subsequent observation of a resistance response, i.e. the induction of HR. The standard procedure of screening for a visual resistance response, i.e. the induction of local necrosis by one of the candidate proteins, was followed in this study. To this end, the *N* and *NSs* genes of RI and RB isolates were initially expressed in *Capsicum* plants carrying the *Tsw* resistance gene using the potato virus x (PVX) expression vector pGR106. At 7 dpi, resistant *Capsicum* plants showed local necrosis with all PVX constructs, including the empty negative control. Furthermore, the systemic symptoms were equal in all plants tested, irrespective of the PVX construct used (Fig. S1, see Supporting Information). Similar results were obtained when, instead of the PVX replicon, a tobacco mosaic virus (TMV) replicon was used to express the *N* and *NSs* genes.

As the viral replicon system appeared to be unsuitable for the identification of the *Avr* gene, an *Agrobacterium*-based transient expression vector system was employed. Initially, various *Agrobacterium tumefaciens* strains (1D1249, AGLO, AGL1, COR308, GV3101 and LBA4404), equipped with the highly translatable binary expression vector pEAQ-HT, were tested for transformation

efficiency and symptom expression in *Capsicum* plants. For easy monitoring, the vector contained a copy of the green fluorescence protein (GFP) gene. All *A. tumefaciens* strains, except 1D1249, induced necrosis starting from 5 dpi. Strain 1D1249 only showed mild chlorosis on extended (>7 dpi) incubation. Furthermore, the presence of the helper plasmid pCH32 increased significantly the transformation efficiency of *Capsicum* leaves. Subsequently, the *N* and *NSs* genes from the RI (Vir129) and RB (Vir171) isolates were cloned into pEAQ-HT and transformed into *Agrobacterium* 1D1249 (+ pCH32). With these constructs, an *A. tumefaciens* transient transformation assay (ATTA) was performed and protein expression was verified and confirmed by Western immunoblot analysis of infiltrated leaf samples (Fig. 3). Transient expression of the *NSs* gene of the RI isolate induced a clear necrosis of the infiltrated area in *Tsw*-containing *Capsicum* plants, visible from 3 dpi [Figs 4 and S2 (see Supporting Information)]. The *NSs* gene from the RB isolate and the *N* genes from the RI and RB isolates only caused mild chlorosis of the infiltrated area, similar to leaves infiltrated with the negative control (empty *Agrobacterium* 1D1249 + pCH32; Fig. 4). A similar chlorosis was observed for all constructs, including NSs from the RI strain, in susceptible *Capsicum* plants (Figs 4 and S2). These results were repeated and confirmed with the *NSs* genes from another RI isolate (Vir127) and another RB isolate (Vir160). To exclude the possibility that the presence of the P19 (RSS from Tombusvirus) protein from the pEAQ-HT vector interfered with HR induction, and that high levels of expression were not involved, the *Avr* protein activity of NSs was also tested after expression from a standard 35S promoting plasmid (pBin19 or pK2), without the P19 protein. Also in this case, and with NSs^{RI} expression levels similar to those of NSs^{RB} from

Fig. 3 Western immunoblot detection of N and NSs proteins expressed from pEAQ-HT in *Nicotiana benthamiana*. Infiltrated *N. benthamiana* leaves were collected at 5 days post-inoculation (dpi). (a) Western blot result of the pEAQ-NSs samples. NSs^{RI} was diluted 10 times in comparison with NSs^{RB}. The positive control here is the TSWV extract (Vir129). (b) Western blot result of the pEAQ-N samples, where the same amounts of sample were loaded in each lane. Here, also, the TSWV extract is used as a positive control. (c) Western blot analysis of NSs^{RB171} expressed from the pEAQ vector compared with NSs^{RI} from the pK2GW7 vector. Similar expression levels can be observed. Here, also, the TSWV extract is used as a positive control. ATTA, *Agrobacterium tumefaciens* transient transformation assay; RB, resistance breaking; RI, resistance inducing.

Fig. 4 Symptoms on resistant and susceptible *Capsicum annuum* leaves infiltrated with various pEAQ-HT constructs. The N and NSs genes derived from resistance-inducing (RI) and resistance-breaking (RB) isolates were transiently expressed in the resistant and susceptible *Capsicum* plants using the pEAQ-HT vector. Leaves were collected at 5 days post-inoculation (dpi) from the resistant *Capsicum* leaves (top row) and susceptible *Capsicum* leaves (bottom row). A hypersensitive response (HR) was only observed with a NSs^{RI} protein gene construct on the resistant *Capsicum* plants, but not on the susceptible plants.

pEAQ-HT (Fig. 3c), an HR was clearly induced at 3–5 dpi in resistant *Capsicum* plants (data not shown).

NSs^{RB} loses its function to trigger HR and its ability to act as RSS

Previously, it has been shown that the TSWV NSs protein possesses RSS activity and, recently, Schnettler *et al.* (2010) have shown that this protein is able to exert this activity most likely by sequestering long dsRNA and short (si and mi)RNAs, thereby preventing cleavage by dicer and uploading into RISC, respectively. The finding that the NSs protein also triggers *Tsw*-induced HR raises the question of whether NSs from the RB isolate still retains the capacity to suppress RNA silencing. To answer this question, NSs^{RB} was tested in a co-ATTA with a sense GFP construct on *N. benthamiana* (Voinnet *et al.*, 1999). As the pEAQ-HT expression vector contains P19, the NSs genes were re-cloned into the binary expression vector pK2GW7 (Karimi *et al.*, 2002) using Gateway technology, and verified for protein expression by Western immunoblot analysis (data not shown). In co-ATTAs of the NSs genes from the RI and RB isolates and a sense GFP construct, silencing of GFP was suppressed by NSs^{RI} at 5 dpi, as indicated by an increase in fluorescence (Fig. 5a). Leaves expressing NSs^{RB} from isolates Vir160 and Vir171 did not show an increase in fluorescence and the protein had apparently lost its RSS capability (Fig. 5a). RSS activity was also estimated quantitatively by measuring the number of fluorescent units from infiltrated leaves (Fig. 5b). Controls consisted of untreated leaves and leaves co-infiltrated with GFP and the negative control maltose-binding protein (MBP) (Schnettler *et al.*, 2010). The amount of fluorescence in leaves infiltrated with NSs^{RI} was approximately four times higher than that recorded in leaves infiltrated with MBP (control), showing RSS activity of the NSs^{RI} protein. In leaves infiltrated with NSs^{RB} constructs, fluorescence levels were similar to those in the MBP control, indicating that the NSs proteins from both RB isolates had lost their RSS capacity (Fig. 5b).

RB viruses are able to suppress local silencing of GFP

As the NSs^{RB} protein had lost its RSS activity, it was questioned whether the RB viruses possessed a reduced fitness in comparison with RI viruses. To address this question, *N. benthamiana* plants were inoculated with two RI and two RB isolates of TSWV and systemically infected leaves were analysed by DAS-ELISA to determine the virus titres. In addition, an antigen-coated plate (ACP)-ELISA was performed on the same leaf material to measure the amount of NSs protein. Surprisingly, no difference in virus or NSs titre was found between the different RI and RB isolates (Fig. 6a,b) and, together with the earlier observation that NSs from the RB virus was not able to suppress GFP silencing in a leaf patch assay, raised the question of whether, during a natural infection, the RB virus was still able to counteract RNAi or evade it in another way.

To test this hypothesis, a local RNA silencing suppression assay was performed, but this time in the presence of a viral infection. During repeated experiments, the results consistently showed that, in contrast with the inability of the RB NSs protein to suppress GFP silencing under transient conditions, the TSWV RB isolates, like the RI isolates, were still able to suppress GFP silencing (Fig. 6c). The absence of a clear GFP silencing suppression in the presence of PVX (Fig. 6c), known to encode a weak RSS (P25), indicated that TSWV (RB) virus was, in some way, still able to suppress RNA silencing.

DISCUSSION

Here, we have shown unambiguously that the NSs protein of TSWV is the *Avr* determinant of *Tsw* gene-based resistance. Although the NSs protein of TSWV RI strains triggered an HR in resistant *Capsicum* at 3–5 dpi, the NSs proteins of RB strains and the N proteins of RI and RB strains did not. Interestingly, the loss of *Avr* activity of the RB NSs protein coincided with a loss of RSS activity. Although plant virus RSSs have been reported previously as *Avr* determinants (Li *et al.*, 1999; Moffett, 2009), the present research shows, for the first time, that the corresponding *Avr* gene from a naturally occurring RB isolate has entirely lost both its *Avr* and RSS activity. In the past, a similar situation has been reported for RB isolates of *Tobacco mild green mosaic virus* (TMGMV) against the *Tm-1* resistance gene from tomato, but, in these cases, the isolates had not lost completely their ability to suppress RNA silencing (Ishibashi *et al.*, 2011). These results not only indicate a putative link between the *Tsw* resistance mechanism and the RNAi pathway, but also positions TSWV NSs as an effector protein in the zigzag model (Jones and Dangl, 2006).

In two previous studies, the identification of the TSWV *Avr* gene for *Tsw*-based resistance has been addressed, with conflicting outcome. Margaria *et al.* (2007) suggested NSs as the *Avr* gene, whereas Lovato *et al.* (2008) identified the TSWV N gene as the *Avr* determinant. Margaria *et al.* based their conclusion merely on the exclusion of other TSWV proteins as candidates for the *Avr* determinant, because attempts to provide actual experimental proof, i.e. to show HR induction by NSs, failed. Lovato *et al.* used a PVX replicon in their assays to express the viral proteins and, as shown here, such viral replicons induce necrotic lesions in *Capsicum*, which can be mistaken for an HR response. A third, more recent, report describes the phylogenetic analysis of different TSWV isolates derived from *Capsicum* plants, in which the authors claim that some mutations observed in the NSs gene were positively selected by the *Tsw* gene (Tentchev *et al.*, 2011). Although this report hints at NSs as the *Avr* determinant of *Tsw*-based resistance, no experimental proof was provided in support of this.

An additional interesting finding from our study was the observation that the NSs protein from the RB isolate lost its RSS activity. Considering that a loss of RSS activity affects the counter defence

Fig. 5 Green fluorescence protein (GFP) silencing suppression assay in *Nicotiana benthamiana* leaves. A co-infiltration of a GFP-expressing construct with pK2GW7-NSs^{RI} (from Vir129) or NSs^{RB} was performed on *N. benthamiana*, and leaves were collected at 5 days post-inoculation (dpi) for GFP monitoring. (a) Images of GFP fluorescence in infiltrated leaves, showing that NSs^{RI} is able to suppress RNA silencing, when compared with the positive control P19, whereas both RI isolates are unable to suppress silencing, when compared with the negative control maltose-binding protein (MBP). (b) The number of fluorescent units measured in a leaf disc (1 cm²) collected from the agroinfiltrated leaf area. Error bars are calculated from an average of six measurements. The data support the result from (a). RB, resistance breaking; RI, resistance inducing.

of the virus against RNAi and, as a consequence, would lead to a reduction in virus titre, it was surprising to see that, for the RI and RB isolates, similar virus titres were detected during infection in *N. benthamiana*, concomitant with the presence of RNA silencing suppression activity, as observed during GFP silencing in a leaf patch assay. These data suggest that either NSs RSS activity is recovered during viral infection (in concert with other viral proteins) or that the virus encodes for another RSS. As NSs expression levels from RB strains during a natural infection were always higher than transient RB NSs expression, but similar to those from RI strains, the possibility that RB NSs still contains some residual

RSS activity that is only observed during enhanced expression levels (during virus infection) and not during lower expression levels (transient) cannot be entirely ruled out. However, as transient expression levels of RB NSs were consistently lower, as also observed with other nonfunctional viral RSS proteins (Díaz-Pendón and Ding, 2008; Schnettler *et al.*, 2008), even from the high expression vector pEAQ-HT, this not only indicated that the protein was affected in its RSS functionality, but also further hampered its transient expression to high levels to analyse for residual RSS activity. In the light of this, it remains to be mentioned that the TSWV RNA-dependent RNA polymerase (L protein; 331 kDa) has

Fig. 6 Enzyme-linked immunosorbent assay (ELISA) measuring virus titres of different *Tomato spotted wilt virus* (TSWV) isolates and their ability to suppress green fluorescence protein (GFP) silencing. (a) NSs titres of TSWV resistance-inducing (RI) and resistance-breaking (RB) isolates were measured by antigen-coated plate (ACP)-ELISA and are shown relative to the virus titres measured by double antibody sandwich (DAS)-ELISA. The average of four repetitions is shown, demonstrating that there is no significant difference between these isolates. (b) Western immunoblot detection of NSs in *Nicotiana benthamiana*, transiently expressed from pEAQ-HT or after virus infection. Transiently expressed NSs shows a difference in amount between the RI (from Vir129) and RB isolates, whereas, in the virus setting, similar amounts can be observed. In addition, in both the transient setting and viral setting, a size difference between the RI and the RB isolates can be observed. ATTA, *Agrobacterium tumefaciens* transient transformation assay. (c) Local transient GFP expression was superimposed with a mechanical TSWV inoculation on the same leaf. Photographs were taken at 5 days post-inoculation (dpi), and show that all isolates are able to suppress local GFP silencing. As a negative control, *Potato virus X* (PVX) was used, which does not show local suppression of GFP silencing.

gene-mediated immunity is a second line of defence that is triggered by effectors. The resulting arms race, nicely illustrated by the zigzag model (Jones and Dangl, 2006), thereby implies a link between RNAi and *R* gene-mediated immunity for viral pathogens with a key role for viral RSS as effectors, but experimental evidence for this has so far been scarce.

Only a few cases have been described in which viral RSSs have also been reported as effectors (Avr determinant), and these are limited to *Tomato bushy stunt virus* (TBSV) P19, *Tomato aspermy virus* (TAV) 2b, PVX 25 K and *Turnip crinkle virus* coat protein (TCV CP) (Angel *et al.*, 2011; Li *et al.*, 1999; Malcuit *et al.*, 1999; Oh *et al.*, 1995; Scholthof *et al.*, 1995). Only for the TAV 2b protein was a clear link found between the Avr and RSS activity (Chen *et al.*, 2008; Li *et al.*, 1999), but not for the TBSV P19 (Hsieh *et al.*, 2009) and TCV CP (Choi *et al.*, 2004).

Although the *Tsw* resistance gene has not yet been cloned, it has been shown to be a single dominant resistance gene (Jahn *et al.*, 2000), and thus most likely of a common nucleotide-binding site and leucine-rich repeat (NBS-LRR) type. Although the TSWV NSs protein is not the first example of an RSS protein that also acts as an Avr determinant, to date it is the first virus for which natural RB isolates have been collected from the field and their respective NSs gene copy has been shown to lack both RSS and Avr activity. Although their functional domains have not yet been mapped, these observations were confirmed for two different RB isolates, which strengthens the idea that RSS and Avr activity within NSs are tightly linked, similar to TAV 2b. Although these findings support the idea that the corresponding *R* genes may be triggered by a functional (RSS) aspect of the Avr determinant, the possibility that this only requires a minimal secondary structural feature cannot be excluded. However, solving this issue might be hampered by the difficulty in separating functions in multifunctional viral proteins and, as a consequence, only functional viral proteins may be recognized by *R* gene products. Another example in

never been tested for RSS activity (Bucher *et al.*, 2003; Takeda *et al.*, 2002). The observation that PVX in the viral setting did not give a clear GFP silencing suppression, whereas its suppressor of RNAi, P25, earlier showed transient RSS activity in this assay (Bayne *et al.*, 2005), supports the idea that the observed RSS activity with TSWV (RB) is not just an artefact.

Nowadays, RNA silencing is well accepted as a virus-triggered immune mechanism in plants and is suppressed by viral RSS proteins, which could alternatively be referred to as effectors. *R*

Fig. 7 Illustration of the zigzag model for the *Tomato spotted wilt virus* (TSWV) pathosystem and the *Tsw* resistance. Representation of the arms race between TSWV and plants containing the *Tsw* resistance gene [modified from Moffett (2009) and Chisholm *et al.* (2006)] in which the roles of NSs as RNA silencing suppressor (RSS) and avirulence (Avr) protein are indicated. The left panel shows the stage of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), represented by the RNA interference (RNAi) response against plant virus infection. The middle panel depicts the stage of a successful infection during which a functional viral RNAi suppressor protein, in this case TSWV NSs, blocks the RNA silencing pathway [preventing cleavage of double-stranded RNA (dsRNA) by dicer-like (DCL) and the uploading of short interfering RNAs (siRNAs) into the RNA-induced silencing complex (RISC)]. The right panel represents the stage of effector-triggered immunity (ETI) during which the NSs is recognized as the Avr determinant and triggers the *Tsw*-induced hypersensitive response (HR).

support of this is the inability to obtain PVY NlaPro mutants that have lost protease activity but still retain the ability to trigger HR by the *Ry* resistance gene (Mestre *et al.*, 2000, 2003).

In past and current literature, examples have been described in which the necrotic response (cell death) induced by effector proteins and resistance against the pathogen are physiological processes that can be separated (Bai *et al.*, 2012; Bendahmane *et al.*, 1999). Nevertheless, to date, HR has generally been accepted as a clear output of activated resistance, also in the case of a systemic HR (SHR), and the invasion of the pathogen is not entirely prevented. The latter is supported by studies on, for example, PVX and the *Rx* resistance gene, where insufficient or partial recognition of the Avr determinant (CP) leads to an SHR (Farnham and Baulcombe, 2006; Tameling and Baulcombe, 2007), and other examples, such as the work of Dinesh-Kumar *et al.* (2000) on the *N* gene from tobacco against TMV and the study of *Plantago asiatica mosaic virus* (PIAMV) in *N. benthamiana* (Komatsu *et al.*, 2011). In our study, we have clearly shown that NSs^{RI} only induces necrosis on *Tsw*-containing plants and not on susceptible plants, which indicates that the induction of HR is linked directly to the presence of the resistance gene and identifies NSs as the Avr determinant. Although Margaria *et al.* (2007) have reported TSWV RB isolates that induce systemic HR, these observations are likely to be

explained as a result of a partial recognition of the Avr determinant, as described above for PVX and *Rx*. The induction of HR is the dominant response, as a co-ATTA with NSs^{RI} and either NSs^{RB160} or NSs^{RB171} from the pEAQ vector on resistant *Capsicum* plants showed an HR (Fig. S3, see Supporting Information).

Interestingly, on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), NSs^{RI} consistently ran at a higher molecular weight than NSs^{RB160/171}, irrespective of transient or viral expression. A closer look at the amino acid sequence of both did not provide any indications for possible post-translational modifications in NSs^{RI} that could have caused this; therefore, the reason for the migration difference remains unresolved to date.

Based on the data, a model is proposed [modified from Chisholm *et al.* (2006) and Moffett (2009)] for the TSWV–*Tsw* pathosystem in which the dual role of NSs as a suppressor of the innate (RNAi) immune system and an Avr determinant for *Tsw*-induced HR is presented (Fig. 7). Whether the RSS function of NSs is coupled to the Avr activity because of an (overlapping) structural conformation, or truly functionally coincides with Avr activity, remains to be resolved by future analysis of additional RB isolates and NSs domain mapping studies. Although the mode of action of resistance genes 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 resistance gene product guards a certain host protein, and is able to perceive alterations of its 'guardee' target on interaction with the Avr determinant, which leads to the induction of HR. Unfortunately, this model does not explain how RB virus isolates preserve their virulence. In our described case of TSWV, however, this might be explained by the presence of an additional viral RSS protein. In addition to the guard model, and to explain the preservation of virulence, other models have been proposed, e.g. the decoy model (van der Hoorn and Kamoun, 2008) or the more recently proposed broader resistance model, the bait and switch model (Collier and Moffett, 2009). Regardless of the model for *Tsw* resistance, the identification of possible host protein target(s) for NSs, whether guardee, decoy or bait, will become the next challenge and contribute to a further unravelling of resistance gene mechanisms.

EXPERIMENTAL PROCEDURES

Virus and plant material

Four different virus isolates of TSWV were included in this study, i.e. Vir127 (Romania, 1998), Vir129 (the Netherlands; WUR, 2002), Vir160 (Spain; Almeria, 2006) and Vir171 (Spain; Almeria, 2008) (Table 1). Virus isolates were maintained on *N. benthamiana* by serial passaging (maximum of five times) using mechanical inoculation (de Avila *et al.*, 1993) and stocked as frozen leaves at -80°C . To confirm the phenotypes of the isolates, two genotypes of *C. annuum* were used: HK0004, a TSWV-susceptible cultivar ($-Tsw$), and YF0009, a TSWV-resistant cultivar ($+Tsw$). *Capsicum chinense* PI 152225 was included as the original *Tsw* source host. All plants were grown and maintained under glasshouse conditions (24°C with a 16 h light/8 h dark regime).

Amplification and sequence verification of *N* and NSs genes

Total RNA was isolated from (infected) leaves using Trizol (Invitrogen, Paisley, UK). From the total RNA, $0.5\text{ }\mu\text{g}$ was used as a template for first-strand cDNA synthesis and subsequent polymerase chain reaction (PCR) amplification of the *N* and NSs genes employing the following primer sets at an annealing temperature of 55°C : *N*-Fw (5'-dGGCGGCCGCATGTCTAAGTTAAG-3') and *N*-Rv (5'-dCCGTCGAC TCAAGCAAGTTCTGC-3'); NSs-Fw (5'-dGGCGGCCGCATGTCTCAAGTGT-3') and NSs-Rv (5'-dCCGTCGACTTATTGTGATCTGAA-3').

For feasible cloning, all forward (Fw) primers also contained a *NotI* restriction site, and all reverse (Rv) primers also contained a *SalI* restriction site, both at the 5' end (highlighted in italic type). PCR amplification was performed using Phusion high-fidelity Taq polymerase, according to the manufacturer's procedures (Finnzymes; Thermo Scientific, Waltham, MA, USA). Amplified DNA products were resolved on a 1% agarose gel and fragments corresponding in size to the *N* and NSs genes were gel purified and subsequently cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Positive clones were selected and verified by sequence analysis. Nucleotide and amino acid sequences from the *N* and NSs genes of the TSWV isolates in this study were analysed by multiple sequence alignment

using the CLUSTALW algorithm. Alignments were edited using the BioEdit program (Hall, 1999). The sequence of TSWV BR01 (GenBank accession D00645) was included as reference isolate.

Cloning procedures

To express the *N* and NSs genes from a PVX replicon, the corresponding genes were excised by *NotI* and *SalI* from pGem-T Easy plasmid and subsequently cloned into *NotI/SalI*-digested pGR106 (Lu *et al.*, 2003). Positive clones were selected and transformed into *Agrobacterium* strain GV3101 (Holsters *et al.*, 1980) containing helper plasmid pSoup (Hellens *et al.*, 2000). An ATTA was performed and the PVX replicon was expressed. The expression of *N* and NSs from the PVX replicon was verified by SDS-PAGE and Western immunoblot analysis of *N. benthamiana* leaf samples collected from local and systemic leaves, at 5 and 7 dpi, infiltrated with *Agrobacterium* containing the PVX replicon constructs. Systemic infected leaves of *N. benthamiana* that scored positive for *N* and NSs expression were used as an inoculum source for the challenge of *Capsicum* plants. For transient expression of TSWV *N* and NSs, the highly translatable pEAQ-HT vector system was used (Sainsbury *et al.*, 2009). To this end, coding sequences for *N* and NSs were re-cloned by *NotI* excision from pGem-T Easy vector constructs into *NotI*-digested pEntr11-*ccdB* (from which the *ccdB* gene was removed by *EcoRI* digestion). Positive clones were selected and verified by sequence analysis, and subsequently used for transfer of the *N/NSs* gene inserts via an LR reaction into a Gateway (Invitrogen)-compatible pEAQ-HT-pDest1 destination vector (Sainsbury *et al.*, 2009). The clones obtained were transformed into *A. tumefaciens* 1D1249 cells, containing helper plasmid pCH32. An ATTA was performed to express the transgenes and the expression was verified by Western immunoblot analysis of leaf samples infiltrated and collected at 5 dpi. Leaves were de-stained in ethanol and acetic acid solution (3:1, v/v) for 5–6 days to visualize the necrotic tissue after induction of the HR.

Agrobacterium transient transformation assay (ATTA)

ATTA was performed according to the protocol of Bucher *et al.* (2003), with slight modifications. In brief, *A. tumefaciens* was grown overnight at 28°C in LB3 medium containing appropriate antibiotic selection pressure. From this culture, $600\text{ }\mu\text{L}$ were freshly inoculated into 3 mL of induction medium and grown overnight. Strain *A. tumefaciens* 1D1249 (Wroblewski *et al.*, 2005) with helper plasmid pCH32 (Hamilton *et al.*, 1996) was grown under $1.25\text{ }\mu\text{g/mL}$ tetracycline selection pressure, whereas *A. tumefaciens* LBA4044 (Ooms *et al.*, 1982) was grown under $20\text{ }\mu\text{g/mL}$ rifampicin selection pressure. Additional strains used during this study were COR308 (Hamilton *et al.*, 1996), selected with $2\text{ }\mu\text{g/mL}$ tetracycline, and AGL0 and AGL1 (Lazo *et al.*, 1991), selected with $20\text{ }\mu\text{g/mL}$ rifampicin and $100\text{ }\mu\text{g/mL}$ carbenicillin, respectively.

Serological detection of virus and proteins

TSWV virus was detected and titres were determined by dot blot and ELISA analysis, respectively. Dot blot analysis was performed on leaf samples from systemically infected *N. benthamiana* leaves (7 dpi) ground in phosphate-buffered saline (PBS)-Tween (0.05% v/v) and spotted onto nitrocellulose in a dilution series of 0, 5, 25 and 125 times. The filter was blocked with 2% fat free milk powder (ELK; Campina, Amersfoort, the

Netherlands) + PBS-Tween (0.05% v/v), washed with 0.25% ELK + PBS-Tween and subsequently incubated with antiserum against TSWV (de Avila *et al.*, 1993). Antigen–antibody complexes were detected with goat–anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Dako, Glostrup, Denmark) nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) as a substrate (Roche, Almere, the Netherlands). Virus titres were analysed by ELISA using antiserum against TSWV. ELISA was performed on systemically infected leaf extracts from *Capsicum* species (10 dpi) and *N. benthamiana* plants (7 dpi) in PBS-Tween buffer (1 : 3, w/v) in a DAS format, according to de Avila *et al.* (1993). Absorbance values were measured at 405 nm using a Fluorstar plate reader (BMG Labtech, Ortenberg, Germany), 30 and 50 min after the addition of the substrate. ACP-ELISA was used to measure the NSs titres employing α NSs as a primary antibody. ACP-ELISA was performed in a similar manner to DAS-ELISA, except that the plates were coated with extracts from systemically infected leaves ground in 2 $\times$ coating buffer (1 L: 3.18 g Na₂CO₃ + 5.86 g NaHCO₃). The expression of TSWV N and NSs proteins was analysed by Western immunoblot analysis using polyclonal antisera against TSWV N and NSs, as described previously (de Avila *et al.*, 1993).

GFP silencing suppression assay

Leaves were agroinfiltrated with a functional GFP construct (Tsien, 1998) as described above, with a final optical density at 600 nm (OD_{600 nm}) of 0.5 per construct. A construct expressing MBP was used as a negative control (Schnettler *et al.*, 2010). Infiltrated leaves were monitored for GFP expression at 5 and 10 dpi 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 fluorescence units was measured using a Fluorstar Optima (BMG Labtech). Suppression of local RNA silencing by virus infection was analysed after agroinfiltration of a functional GFP construct and subsequent mechanical inoculation of the same leaf area with each of the TSWV isolates and PVX, as described earlier. The results were observed at 5 dpi.

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REFERENCES

- Alvarado, V. and Scholthof, H.B. (2009) Plant responses against invasive nucleic acids: RNA silencing and its suppression by plant viral pathogens. *Semin. Cell. Dev. Biol.* **20**, 1032–1040.
- 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.
- Aramburu, J. and Marti, M. (2003) The occurrence in north-east Spain of a variant of *Tomato spotted wilt virus* (TSWV) that breaks resistance in tomato (*Lycopersicon esculentum*) containing the *Sw-5* gene. *Plant Pathol.* **52**, 407.
- de Avila, A.C., Huguenot, C., Resende, R.O., Kitajima, E.W., Goldbach, R.W. and Peters, D. (1990) Serological differentiation of 20 isolates of *Tomato spotted wilt virus*. *J. Gen. Virol.* **71**, 2801–2807.
- de Avila, A.C., de Haan, P., Smeets, M.L.L., Resende, R.D., Kormelink, R., Kitajima, E.W., Goldbach, R.W. and Peters, D. (1993) Distinct levels of relationships between tospovirus isolates. *Arch. Virol.* **128**, 211–227.
- Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., Xiao, W., Liu, Y., Chai, J., Takken, F.L., Schulze-Lefert, P. and Shen, Q.H. (2012) Structure–function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. *PLoS Pathog.* **8**, e1002752.
- Bayne, E.H., Rakitina, D.V., Morozov, S.Y. and Baulcombe, D.C. (2005) Cell-to-cell movement of Potato potexvirus X is dependent on suppression of RNA silencing. *Plant J.* **44**, 471–482.
- Bendahmane, A., Kanyuka, K. and Baulcombe, D.C. (1999) The *Rx* gene from potato controls separate virus resistance and cell death responses. *Plant Cell*, **11**, 781–792.
- 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.
- Black, L.L., Hobbs, H.A. and Gatti, J.M. (1991) *Tomato spotted wilt virus* resistance in *Capsicum chinense* PI152225 and 159236. *Plant Dis.* **75**, 863.
- Boiteux, L.S. (1995) Allelic relationships between genes for resistance to *Tomato spotted wilt tospovirus* in *Capsicum chinense*. *Theor. Appl. Genet.* **90**, 146–149.
- Boiteux, L.S. and de Avila, A.C. (1994) Inheritance of a resistance specific to *Tomato spotted wilt tospovirus* in *Capsicum chinense* PI159236. *Euphytica*, **75**, 139–142.
- Boiteux, L.S. and de B. Giordano, L. (1993) Genetic basis of resistance against two *Tospovirus* species in tomato (*Lycopersicon esculentum*). *Euphytica*, **71**, 151–154.
- 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.
- Chisholm, S.T., Coaker, G., Day, B. and Staskawicz, B.J. (2006) Host–microbe interactions: shaping the evolution of the plant immune response. *Cell*, **124**, 803–814.
- 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.
- Collier, S.M. and Moffett, P. (2009) NB-LRRs work a 'bait and switch' on pathogens. *Trends Plant Sci.* **14**, 521–529.
- Corbà, T., Pantaleo, V. and Burgyn, J. (2009) RNA silencing: an antiviral mechanism. *Adv. Virus Res.* **75**, 35–71.
- Diaz-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.
- Dinesh-Kumar, S.P., Tham, W.H. and Baker, B.J. (2000) Structure–function analysis of the *Tobacco mosaic virus* resistance gene *N*. *Proc. Natl. Acad. Sci. USA*, **97**, 14 789–14 794.
- Ding, S.-W. and Voinnet, O. (2007) Antiviral immunity directed by small RNAs. *Cell*, **130**, 413–426.
- Farnham, G. and Baulcombe, D.C. (2006) Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. *Proc. Natl. Acad. Sci. USA*, **103**, 18 828–18 833.
- Giner, A., Lakatos, L., García-Chapa, M., López-Moya, J.J. and Burgyn, 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.
- Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* **42**, 819–832.
- Holsters, M., Silva, B., Van Vliet, F., Genetello, C., De Block, M., Dhaese, P., Depicker, A., Inzé, D., Engler, G., Villarroel, R., Van Montagu, M. and Schell, J. (1980) The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. *Plasmid*, **3**, 212–230.
- van der Hoorn, R.A.L. and Kamoun, S. (2008) From guard to decoy: a new model for perception of plant pathogen effectors. *Plant Cell*, **20**, 2009–2017.
- 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.
- Ishibashi, K., Meshi, T. and Ishikawa, M. (2011) Gaining replicability in a nonhost compromises the silencing suppression activity of *Tobacco mild green mosaic virus* in a host. *J. Virol.* **85**, 1893–1895.

- Jahn, M., Paran, I., Hoffmann, K., Radwanski, E.R., Livingstone, K.D., Grube, R.C., Aftergoot, E., Lapidot, M. and Moyer, J. (2000) Genetic mapping of the *Tsw* locus for resistance to the Tospovirus *Tomato spotted wilt virus* in *Capsicum* spp. and its relationship to the *Sw-5* gene for resistance to the same pathogen in tomato. *Mol. Plant-Microbe Interact.* **13**, 673–682.
- 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.
- Komatsu, K., Hashimoto, M., Maejima, K., Shiraishi, T., Neriya, Y., Miura, C., Minato, N., Okano, Y., Sugawara, K., Yamaji, Y. and Namba, S. (2011) A necrosis-inducing elicitor domain encoded by both symptomatic and asymptomatic *Plantago asiatica mosaic virus* isolates, whose expression is modulated by virus replication. *Mol. Plant-Microbe Interact.* **24**, 408–420.
- Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.P., Dolja, V.V., Calvino, L.F., Lopez-Moya, J.J. and Burgyn, J. (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J.* **25**, 2768–2780.
- Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Nat. Biotechnol.* **9**, 963–967.
- 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.
- 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.
- Lu, R., Malcuit, I., Moffett, P., Ruiz, M.T., Peart, J., Wu, A.J., Rathjen, J.P., Bendahmane, A., Day, L. and Baulcombe, D.C. (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J.* **22**, 5690–5699.
- 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. and Turina, M. (2004) Resistance breaking strain of *Tomato spotted wilt virus* (Tospovirus; Bunyaviridae) on resistant pepper cultivars in Almeria, Spain. *Plant Pathol.* **53**, 795.
- 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.
- Mestre, P., Brigneti, G. and Baulcombe, D.C. (2000) An *Ry*-mediated resistance response in potato requires the intact active site of the Nla proteinase from *Potato virus Y*. *Plant J.* **23**, 653–661.
- Mestre, P., Brigneti, G., Durrant, M.C. and Baulcombe, D.C. (2003) *Potato virus Y* Nla protease activity is not sufficient for elicitation of *Ry*-mediated disease resistance in potato. *Plant J.* **36**, 755–761.
- Moffett, P. (2009) Mechanisms of recognition in dominant *R* gene mediated resistance. *Adv. Virus Res.* **75**, 1–33.
- Nicaise, V., Roux, M. and Zipfel, C. (2009) Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol.* **150**, 1638–1647.
- 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-Tuin, T.J.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.
- Roggero, P., Masenga, V. and Tavella, L. (2002) Field isolates of *Tomato spotted wilt virus* overcoming resistance in pepper and their spread to other hosts in Italy. *Plant Dis.* **86**, 950–954.
- 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., Goldbach, R. and Prins, M. (2008) The NS3 protein of rice hoja blanca virus suppresses RNA silencing in mammalian cells. *J. Gen. Virol.* **89**, 336–340.
- 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.
- Scholthof, K.-B.G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., Hemenway, C. and Foster, Gary D. (2011) Top 10 plant viruses in molecular plant pathology. *Mol. Plant Pathol.* **12**, 938–954.
- Stevens, M.R., Scott, S.J. and Gergerich, R.C. (1992) Inheritance of a gene for resistance to *Tomato spotted wilt virus* (TSWV) from *Lycopersicon peruvianum* Mill. *Euphytica*, **59**, 9–17.
- 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 Letters* **532**, 75–79.
- Tameling, W.I. and Baulcombe, D.C. (2007) Physical association of the NB-LRR resistance protein *Rx* with a Ran GTPase-activating protein is required for extreme resistance to *Potato virus X*. *Plant Cell*, **19**, 1682–1694.
- Tentchev, D., Verdin, E., Marchal, C., Jacquet, M., Aguilar, J.M. and Moury, B. (2011) Evolution and structure of *Tomato spotted wilt virus* populations: evidence of extensive reassortment and insights into emergence processes. *J. Gen. Virol.* **92**, 961–973.
- Tsien, R.Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544.
- Vargason, J.M., Szitty, G., Burgyn, J. and Hall, T.M.T. (2003) Size selective recognition of siRNA by an RNA silencing suppressor. *Cell*, **115**, 799–811.
- Voinnet, O., Pinto, Y.M. and Baulcombe, D.C. (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. USA*, **96**, 14 147–14 152.
- Wroblewski, T., Tomczak, A. and Michelmore, R. (2005) Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnol. J.* **3**, 259–273.

SUPPORTING INFORMATION

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

Fig. S1 Systemic infection of *Capsicum chinense* by *Potato virus X* (PVX) recombinants. Recombinant PVX constructs carrying the *N^{RI}* or the *NSs^{RI}* gene were inoculated on resistant *C. chinense* plants using an empty PVX vector as a negative control. As visible in all three panels, PVX-induced necrosis covers most of the systemically infected leaves, irrespective of the gene insertion. RI, resistance inducing.

Fig. S2 De-stained leaves showing transient expression of candidate *avirulence* (*Avr*) genes on resistant and susceptible *Capsicum annuum* plants. The leaves from Fig. 4, showing the local response of resistant and susceptible *C. annuum* plants after agroinfiltration of the candidate *Avr* genes *N^{RI/IRB}* and *NSs^{RI/IRB}*, expressed from the pEAQ-HT vector, were de-stained to better visualize the necrotic area (hypersensitive response, HR). Clearly, only the entire infiltrated area of the *NSs^{RI}* sample is completely necrotic, whereas, in the other leaves, some local wounding of the leaf epidermis (allowing easy infiltration of the *Agrobacterium* culture) can be seen. RB, resistance breaking; RI, resistance inducing.

Fig. S3 Triggering of the hypersensitive response (HR) on co-*Agrobacterium tumefaciens* transient transformation assay (ATTA) of *NSs^{RI}* with *NSs^{RB160/171}* on resistant *Capsicum* plants. Leaves of resistant *Capsicum annuum* plants were infiltrated with *NSs^{RI}*, *NSs^{RB160}* and *NSs^{RB171}* alone and in a co-ATTA setting. Photographs were taken at 5 days post-inoculation (dpi) and show an HR in those leaves in which *NSs^{RI}* was expressed.