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The *Tomato spotted wilt virus* cell-to-cell movement protein (NS_M) triggers a hypersensitive response in *Sw-5*-containing resistant tomato lines and in *Nicotiana benthamiana* transformed with the functional *Sw-5b* resistance gene copy

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SUMMARY

Although the *Sw-5* gene cluster has been cloned, and *Sw-5b* has been identified as the functional gene copy that confers resistance to *Tomato spotted wilt virus* (TSWV), its avirulence (*Avr*) determinant has not been identified to date. *Nicotiana tabacum* 'SR1' plants transformed with a copy of the *Sw-5b* gene are immune without producing a clear visual response on challenge with TSWV, whereas it is shown here that *N. benthamiana* transformed with *Sw-5b* gives a rapid and conspicuous hypersensitive response (HR). Using these plants, from all structural and non-structural TSWV proteins tested, the TSWV cell-to-cell movement protein (NS_M) was confirmed as the *Avr* determinant using a *Potato virus X* (PVX) replicon or a non-replicative pEAQ-HT expression vector system. HR was induced in *Sw-5b*-transgenic *N. benthamiana* as well as in resistant near-isogenic tomato lines after agroinfiltration with a functional cell-to-cell movement protein (NS_M) from a resistance-inducing (RI) TSWV strain (BR-01), but not with NS_M from a *Sw-5* resistance-breaking (RB) strain (GRAU). This is the first biological demonstration that *Sw-5*-mediated resistance is triggered by the TSWV NS_M cell-to-cell movement protein.

Keywords: avirulence, cell-to-cell movement, NS_M, resistance gene, *Sw-5*, tospovirus, TSWV.

INTRODUCTION

Tospoviruses are amongst the most destructive pathogens known for tomatoes and are a limiting factor in vegetable production worldwide (Aramburu and Marti, 2003; Boiteux and Giordano, 1993; Finetti Sialer *et al*., 2002; Pappu *et al*., 2009; Williams *et al*., 2001). *Tomato spotted wilt virus* (TSWV) is the best studied member of the genus *Tospovirus* and represents a great burden to many economically important agricultural and ornamental crops in (sub)tropical and temperate climatic regions. The virus is transmitted in a propagative manner by various species of thrip (Thripidae, main vector—*Frankliniella occidentalis*), and currently ranks second on the list of the most important plant viruses in the world (Scholthof *et al*., 2011).

Since TSWV was identified and classified as the first phytopathogenic virus in the *Bunyaviridae*, a large family of mostly arthropod-borne animal-infecting RNA viruses, more than 20 new tospovirus species have been identified and distinguished on the basis of the nucleoprotein (N) gene sequence and vector specificity (King *et al*., 2011). Based on phylogenetic analysis of the N gene, all tospovirus species are grouped into two major clusters that correlate with their geographical distribution, i.e. the New World and Old World tospoviruses. Like all members of the *Bunyaviridae*, tospoviruses consist of enveloped virus particles (Ø 80–120 nm) and contain three RNA segments which, according to their size, are denoted S (small), M (medium) and L (large) RNA. The S RNA encodes the N protein and the RNA silencing suppressor protein NS_S (Bucher *et al*., 2003; Kormelink *et al*., 1991; Takeda *et al*., 2002). The M RNA encodes the glycoprotein precursor (GP) to the glycoproteins G_N and G_C (N and C refer to the amino- and carboxy-terminal positions in the precursor) and the cell-to-cell movement protein NS_M (Kormelink *et al*., 1992, 1994; Storms *et al*., 1995). The L RNA encodes the viral polymerase (de Haan *et al*., 1991).

Several strategies for the chemical and cultural control of tospoviruses and their vectors have been shown to be ineffective. The most successful, however, is the cultivation of resistant cultivars, which are usually developed by the introgression of resistance (*R*) genes into commercial genotypes. Some tomato accessions are sources of natural resistance to TSWV, and are being used in breeding programmes throughout the world (Finlay, 1953; Price *et al*., 2007; Saidi and Warade, 2008). In recent

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decades, two single dominant *R* genes have received most attention because of their applicability for commercial resistance breeding against tospoviruses, i.e. *Sw-5* and *Tsw*. The first, *Sw-5* (Aramburu *et al*., 2011; Cho *et al*., 1995; Stevens *et al*., 1991), is the most interesting as it confers a broad tospovirus resistance against TSWV, *Chrysanthemum stem necrosis virus* (CSNV), *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV) (Boiteux and Giordano, 1993; Stevens *et al*., 1991, 1994, 1995). The resistance derives from a tomato cultivar (Stevens), which was obtained in South Africa by a cross between *Solanum peruvianum* and *S. lycopersicum* (Van Zijl *et al*., 1985), and protects against virus invasion by the induction of a hypersensitive response (HR). Little is yet known about the mechanism of defence driven by this *R* gene. The second, single dominant, *R* gene is *Tsw* (Boiteux, 1995). This gene originates from distinct *Capsicum chinense* accessions and is highly specific as it only confers resistance against TSWV isolates (Boiteux, 1995).

Single dominant *R* genes generally make up the second line of defence of the plant immune system against pathogens, a battle that is generally illustrated with the zig–zag model (Jones and Dangl, 2006) and involves RNA silencing as one of the first lines of defence against plant viruses. The first lines of defence are triggered by the so-called microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Nürnberger and Brunner, 2002), which are perceived by pattern recognition receptors (PRRs) (Postel and Kemmerling, 2009) and lead to the onset of PAMP-triggered immunity (PTI) (Chisholm *et al*., 2006). Viruses encode virulence factors (effectors) that counteract PTI, and thereby enable them to achieve a successful infection. In the next phase, these same effectors are specifically recognized directly or indirectly by protein products from *R* genes, referred to as effector-triggered immunity (ETI). This recognition generally leads to a rapid HR, and involves programmed cell death (PCD) at the site of infection. Recently, the RNA silencing suppressor NS_S has been identified as the effector triggering *Tsw*-mediated resistance (Ronde *et al*., 2013), but the effector that triggers *Sw-5b*-governed resistance has remained unknown to date.

Proteins encoded by single dominant *R* genes typically contain a nucleotide-binding leucine-rich repeat (NB-LRR) domain (Dangl and Jones, 2001). In plants, the NB-LRR-type *R* genes are further divided into two groups based on the structure of the conserved N-terminal domain: TIR-NB-LRRs, which share homology to Toll/interleukin receptors, and the coiled coil (CC)-NB-LRRs (Pan *et al*., 2000). *Sw-5* belongs to the CC-NB-LRR group and its locus contains at least six paralogues, denoted *Sw-5a* to *Sw-5f* (Folkertsma *et al*., 1999; Rehman *et al*., 2009). The *Sw-5b* gene represents the functional resistance gene copy and is able to provide broad tospovirus resistance, as demonstrated in transgenic tobacco plants expressing *Sw-5b* (Spassova *et al*., 2001).

The possible occurrence of genome rearrangements and the ongoing evolution of new TSWV variants by mutations pose a constant threat to the broad resistance against tospoviruses conferred by the *Sw-5* gene. Some natural field isolates of TSWV able to breakdown *Sw-5* resistance have already been reported in tomato crops in Hawaii, Australia, South Africa (JF1 isolate), Spain (GRAU isolate) and Italy (Aramburu and Marti, 2003; Cho *et al*., 1995; Ciuffo *et al*., 2005; Latham and Jones, 1998; Thompson and Van Zijl, 1995). Studies on TSWV reassortants have indicated that the genetic determinant involved in overcoming the resistance is associated with the M RNA (Hoffmann *et al*., 2001). More recently, López *et al*. (2011) proposed, by *in silico* analysis, that two amino acid substitutions in the NS_M protein of TSWV could be responsible for overcoming the resistance of *Sw-5*, but no experimental evidence was provided to support this assumption.

In the present study, the TSWV cell-to-cell movement protein (NS_M) has been identified as the *Avr* determinant for the *Sw-5* resistance gene in *Nicotiana benthamiana* transformed with the functional *Sw-5b* copy and in resistant near-isogenic tomato lines. In addition, a tospovirus challenge assay has shown that *Sw-5*-mediated resistance also protects against the more distinct *Impatiens necrotic spot virus* (INSV), demonstrating its broad resistance profile against several tospovirus species.

RESULTS

Nicotiana benthamiana transformed with *Sw-5b* shows HR on challenge with a TSWV resistance-inducing (RI) isolate

Previously, the *Sw-5b* resistance gene copy was identified as the functional resistance gene, as *Nicotiana tabacum* 'SR1' transformed with this copy conferred resistance against TSWV (Spassova *et al*., 2001). These transgenic lines, however, did not reveal a clear HR and appeared totally immune, which hampered the identification of the TSWV *Avr* determinant of *Sw-5*. To determine whether transformation of another host with *Sw-5b* would provide similar results or, in contrast, would reveal a visual HR and thereby allow the identification of the TSWV *Avr* determinant, *N. benthamiana* was transformed and subsequently analysed for an HR. Although TSWV BR-01 (RI) and GRAU (RB, resistance breaking) both produced a systemic infection on untransformed *N. benthamiana* (Fig. 1A,B), isolate BR-01 (RI) triggered an HR within 3–4 days, as visualized by necrotic lesions only on inoculated leaves of transformed *N. benthamiana* [Fig. 1, photographs in C and D were taken at 5–7 days post-inoculation (dpi) and in E at 15 dpi], indicative of an *Sw-5b*-induced resistance response. Challenge with TSWV-RB isolate GRAU did not trigger an HR-like response, but, instead, resulted in a systemic infection, as in untransformed *N. benthamiana* (Fig. 1F,G). Transformed *N. benthamiana* was additionally challenged with INSV, belonging to the same phylogenetic clade of American tospoviruses, and *Melon yellow spot virus* [MYSV, *Physalis severe mottle virus*

(PSMV) isolate], another more distinct tospovirus from the Eur-Asian clade. Although MYSV systemically infected *Sw-5b*-transformed *N. benthamiana*, INSV surprisingly triggered an HR, as observed with TSWV-RI, suggesting that the spectrum of *Sw-5b*-mediated resistance covers a large range of members from the 'American' tospovirus clade (Fig. S1, see Supporting Information). Although no systemic symptoms were observed in *Sw-5b*-transformed *N. benthamiana* on challenge with TSWV-RI and INSV, the absence of virus from the upper (non-inoculated) leaves was verified by reverse transcription-polymerase chain reaction (RT-PCR) at 10 dpi using systemically infected wild-type *N. benthamiana* as a positive control. As expected, viral RNA was detected in wild-type plants and in *Sw-5b*-transformed *N. benthamiana* inoculated with TSWV-RB or MYSV, but not on challenge with RI tospoviruses TSWV and INSV (Fig. 1H).

Transient expression of TSWV genes from a non-replicative pEAQ-HT and from a *Potato virus X* (PVX) replicon identifies NS_M as the TSWV *Avr* determinant of *Sw-5b*

Earlier studies to identify the TSWV *Avr* determinant that triggers *Tsw*-governed resistance resulted in different and conflicting reports (Lovato *et al*., 2008; Margaria *et al*., 2007). Recent analysis unambiguously identified the TSWV RNA silencing suppressor NS_S as the *Avr* determinant (Ronde *et al*., 2013), but this was only successful with the use of a non-replicative transient pEAQ-HT expression vector (Sainsbury *et al*., 2009), but not when using PVX. To identify the *Avr* determinant, genes coded from TSWV M RNA (NS_M and GP) and possible candidates for the *Avr*-determinant of *Sw-5* were cloned into both PVX (pGR107) and pEAQ-HT (Fig. 2) for agroinfiltration into *Sw-5b*-transformed *N. benthamiana*. Cloning of the GP into pGR107 failed, probably because of size constraints and stability. As controls, constructs for the S RNA-encoded N and NS_S genes were included (Fig. 2; Ronde *et al*., 2013). All gene constructs were verified for their translatability from pEAQ-HT or PVX during agroinfiltration of the constructs into untransformed *N. benthamiana* and subsequent Western immunoblot analysis. Although all NS_M (Fig. 3C), NS_S and N gene constructs (Ronde *et al*., 2013) were well expressed, the viral glycoproteins G_N and G_C processed from GP could not be detected (data not shown), probably because of the low transient expression levels, as witnessed earlier (D. Ribeiro, personal communication).

Although, as expected, a visual HR was observed on challenge of *Sw-5b*-transformed *N. benthamiana* with TSWV BR-01 (RI), and a systemic infection with TSWV GRAU (RB) (Fig. 1), repeated analysis consistently revealed a dark brownish necrosis, typical of an (upcoming) HR-like response, only in leaves infiltrated with pEAQ-HT-$NS_{M\text{-}BR\text{-}01}$ or pGR107+$NS_{M\text{-}BR\text{-}01}$ (Fig. 3). Although photographs were taken at 5 and 6 dpi for better digital images, the HR-like response was already observed at 3 dpi. This necrosis became more distinct later, but was not observed with pEAQ-HT-$NS_{M\text{-}GRAU}$ or pGR107+$NS_{M\text{-}GRAU}$ (Fig. 3A,B). The last two constructs revealed only a weak chlorosis after agroinfiltration of *Sw-5b*-transgenic *N. benthamiana*, similar to that caused by pEAQ-HT or pGR107 without any insert (Fig. 3A,B), or containing GP, N or NS_S from the RI BR-01 strain, or just blank *Agrobacterium tumefaciens*. On chlorophyll removal of agroinfiltrated leaves, the HR-like response after infiltration with $NS_{M,BR\text{-}01}$ was even more distinct (Fig. 3A,B). Infiltration of $NS_{M\text{-}BR\text{-}01}$ and $NS_{M\text{-}GRAU}$ constructs on leaves from untransformed *N. benthamiana* only revealed a weak interveinal chlorosis (Fig. S2, see Supporting Information).

Induction of HR in *Sw*-5 near-isogenic tomato lines

To further substantiate the observation that NS_M represented the *Avr* determinant for *Sw5-b*-mediated resistance in a natural and commercial host fashion, a tomato breeding isoline (CNPH 'LAM 147') harbouring the *Sw*-5 gene was agroinfiltrated with pEAQ-HT constructs of NS_M^{RI} and NS_M^{RB}. At 4 dpi, leaves infiltrated with NS_M^{RI} showed a clear HR, whereas those infiltrated with NS_M^{RB} did not, which was more evident after chlorophyll removal from the leaves (Fig. 4).

DISCUSSION

During earlier studies in which *N. tabacum* 'SR1' was transformed with *Sw-5* resistance gene candidates, *Sw-5b* was identified as the functional resistance gene copy (Spassova *et al*., 2001). However, in these transgenic lines, no clear HR was observed on challenge with TSWV, and transgenic plants were totally immune. Here, we have successfully generated transgenic *N. benthamiana* containing *Sw-5b* that responded with an HR on challenge with the RI TSWV BR-01 (wild-type reference strain), but not with the RB TSWV GRAU isolate (Aramburu and Marti, 2003). Although initially not reported for transgenic *N. tabacum* (Spassova *et al*., 2001), challenge of the *Sw-5b*-transgenic *N. benthamiana* also yielded resistance to GRSV and TCSV (data not shown), two tospovirus species phylogenetically close to TSWV and already reported to induce *Sw-5* resistance (Boiteux and Giordano, 1993). Surprisingly, protection was also observed against INSV, but not against MYSV, which belongs to a distant phylogenetic lineage of the Eur-Asian tospoviruses (de Oliveira *et al*., 2012), indicating that *Sw-5b* resistance acts against a broad spectrum of related tospoviruses belonging to the American tospovirus clade.

Using the *Sw-5b*-transformed *N. benthamiana*, and confirmed with a near-isogenic tomato line, it was shown that *Sw-5b*-governed resistance is triggered by the NS_M cell-to-cell movement protein and not by the (precursor to the) glycoproteins, both candidates for the avirulence determinant (Hoffmann *et al*., 2001), as visualized by the induction of HR after expression of NS_M from the RI TSWV BR-01 isolate, but not from the RB TSWV GRAU

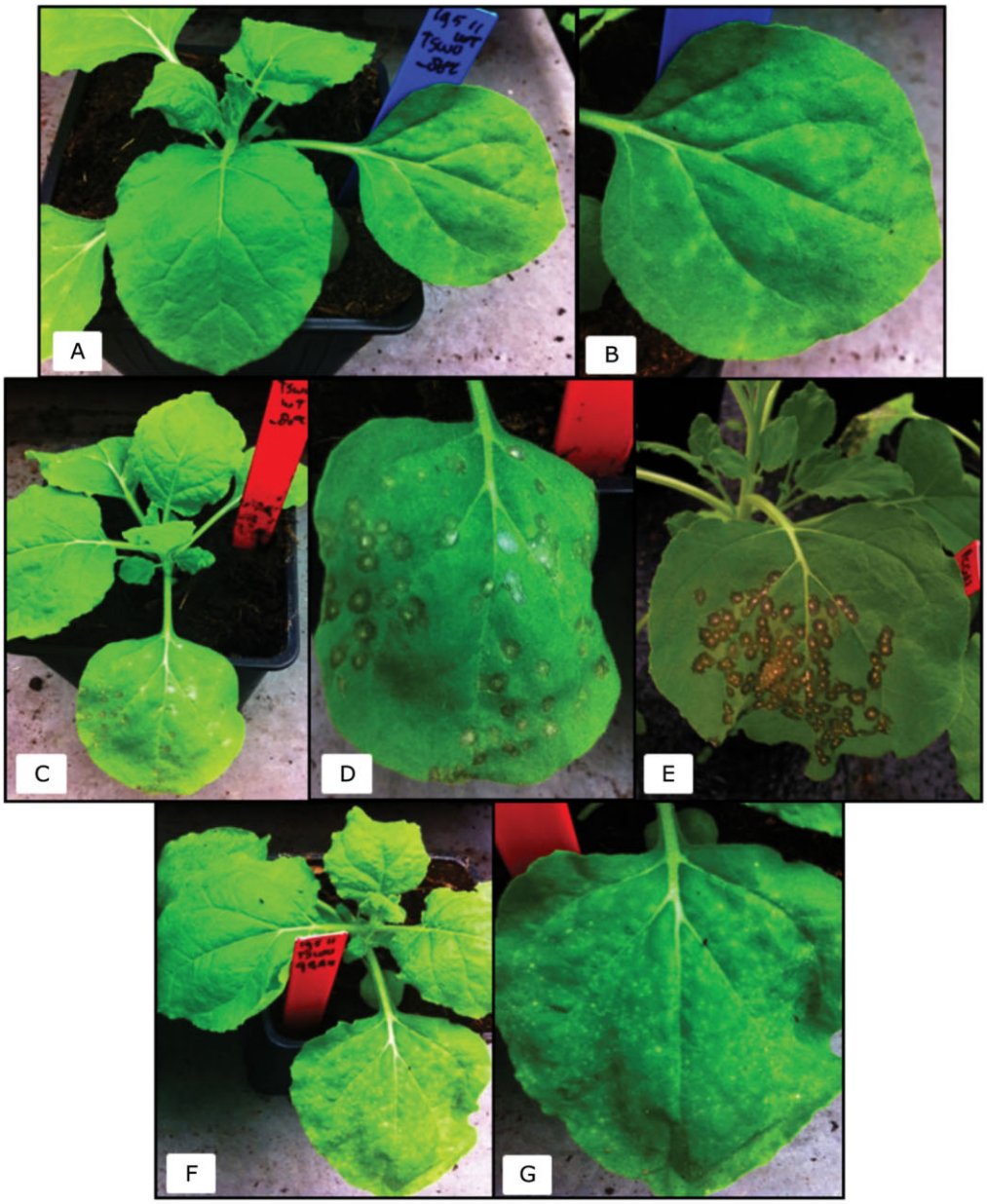

Fig. 1 Challenge of untransformed and *Sw-5b*-transformed *Nicotiana benthamiana* with resistance-inducing *Tomato spotted wilt virus* (TSWV) BR-01 and resistance-breaking TSWV GRAU isolates. Photographs from all leaves were taken at 5–7 days post-inoculation (dpi), with the exception of those shown in (E). (A) Untransformed *N. benthamiana* plant challenged with TSWV BR-01 and showing typical chlorotic lesions on locally infected leaves. (B) Enlarged view of a locally infected leaf from (A). (C) *Nicotiana benthamiana Sw-5b* challenged with TSWV BR-01. (D) Enlarged view of the virus-challenged leaf from (C). (E) *Nicotiana benthamiana Sw-5b* challenged with TSWV BR-01 showing the challenged leaf at 15 dpi. (F) *Nicotiana benthamiana Sw-5b* challenged with TSWV GRAU. (G) Enlarged view of the virus-challenged leaf from (F). (H) Reverse transcription-polymerase chain reaction (RT-PCR) for monitoring systemic infection in wild-type (wt) and *Sw-5b N. benthamiana* plants inoculated with TSWV (BR-01 and GRAU), *Impatiens necrotic spot virus* (INSV) and *Melon yellow spot virus* (MYSV). Total RNA of non-inoculated upper leaves was used as a template for N (nucleocapsid) gene amplification at 10 dpi. M, GeneRuler 100-bp Plus DNA ladder.

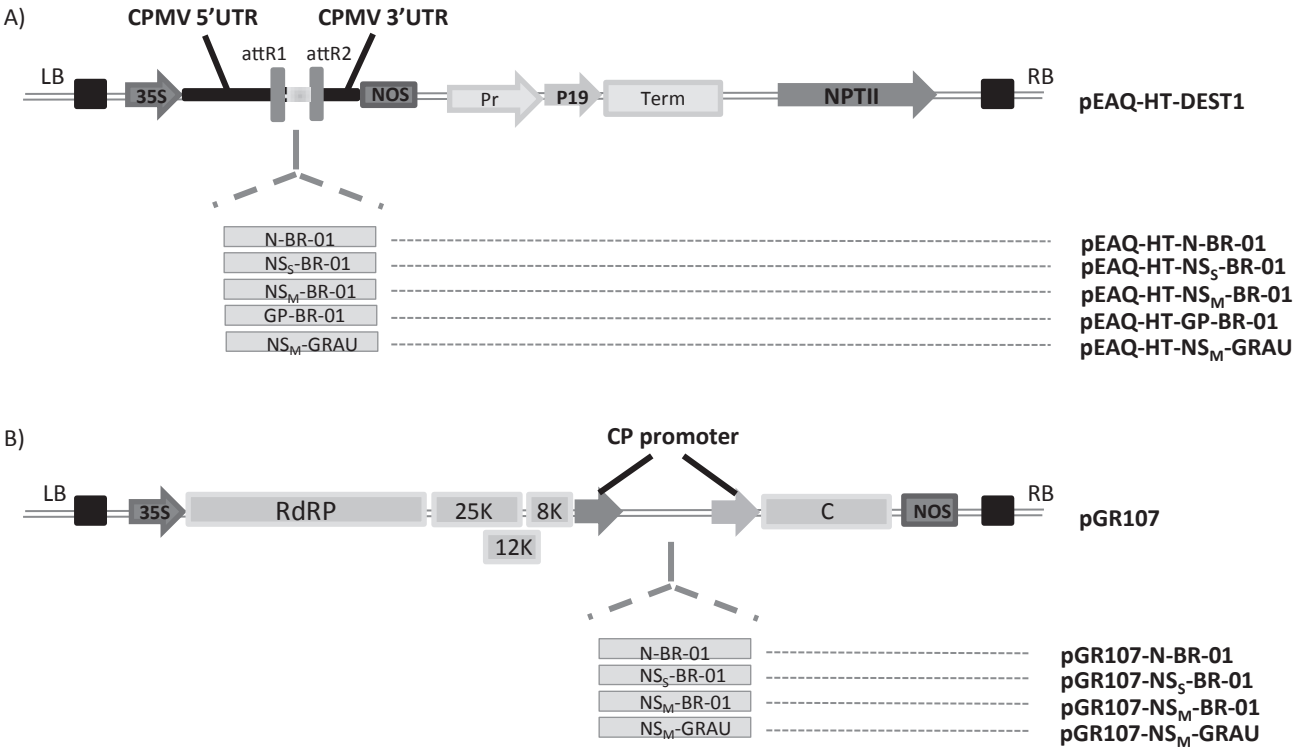

Fig. 2 Schematic representation of the expression vector constructs used to challenge *Nicotiana benthamiana* transformants containing *Sw-5b*. (A) *Tomato spotted wilt virus* (TSWV) genes (NS_M of BR-01 and GRAU isolates; *N*, NS_S and the glycoprotein precursor (GP) from BR-01) cloned individually into the pEAQ-HT vector. AttR1/R2, Gateway recombination sites. (B) *Tomato spotted wilt virus* (TSWV) genes (NS_M of BR-01 and GRAU isolate, *N*, NS_S) cloned individually in the appropriated restriction sites (*Cla*I and *Sal*I) of the pGR107 vector (Lovato *et al*., 2008). LB, left border; RB, right border; 35S, *Cauliflower mosaic virus* promoter region; RdRP, *Potato virus X* (PVX) RNA-dependent RNA polymerase; 25K, 12K and 8K, PVX movement proteins (triple gene block); CP promoter, PVX coat protein subgenomic RNA promoter; CP, PVX coat protein; NOS, nopaline synthase terminator.

isolate. Neither gene triggered a response when agroinfiltrated into untransformed *N. benthamiana* (Fig. S1). In contrast with earlier work on the identification of the NS_S RNA silencing suppressor as the *Avr* determinant of the single dominant *Tsw* resistance gene (Ronde *et al*., 2013), NS_M was able to induce *Sw-5b*-mediated HR when expressed not only from the non-replicative pEAQ-HT vector system (Sainsbury *et al*., 2009), but also from a PVX replicon (pGR107).

An HR response is not restricted to *R*-gene-mediated defence, as it can also result from basal defence and non-host resistance (Somssich and Hahlbrock, 1998), and can be uncoupled from the resistance response conferred by the *R* gene (Bendahmane *et al*., 1999); however, in general, it is activated after *R* genes have been triggered and is thereby used as an indirect indicator for *R*-gene activation. The latter is assumed to involve direct or indirect interactions between the plant resistance gene product (*R*) and the pathogen avirulence factor (*Avr*) (Morel and Dangl, 1997). So far, only a small number of single dominant resistance genes against plant viruses have been cloned, and for these, as well as others for which the *R* genes have not yet been cloned, the polymerase protein (Kim and Palukaitis, 1997; Les Erickson *et al*., 1999), movement protein (Weber *et al*., 1993; Yoshikawa *et al*., 2006), coat protein (Asurmendi *et al*., 2004; Bendahmane *et al*., 2002) and RNA silencing suppressor (Li *et al*., 1999; Malcuit *et al*., 1999; Oh, 1995; Román *et al*., 2011; Ronde *et al*., 2013) have been identified as the *Avr* determinant.

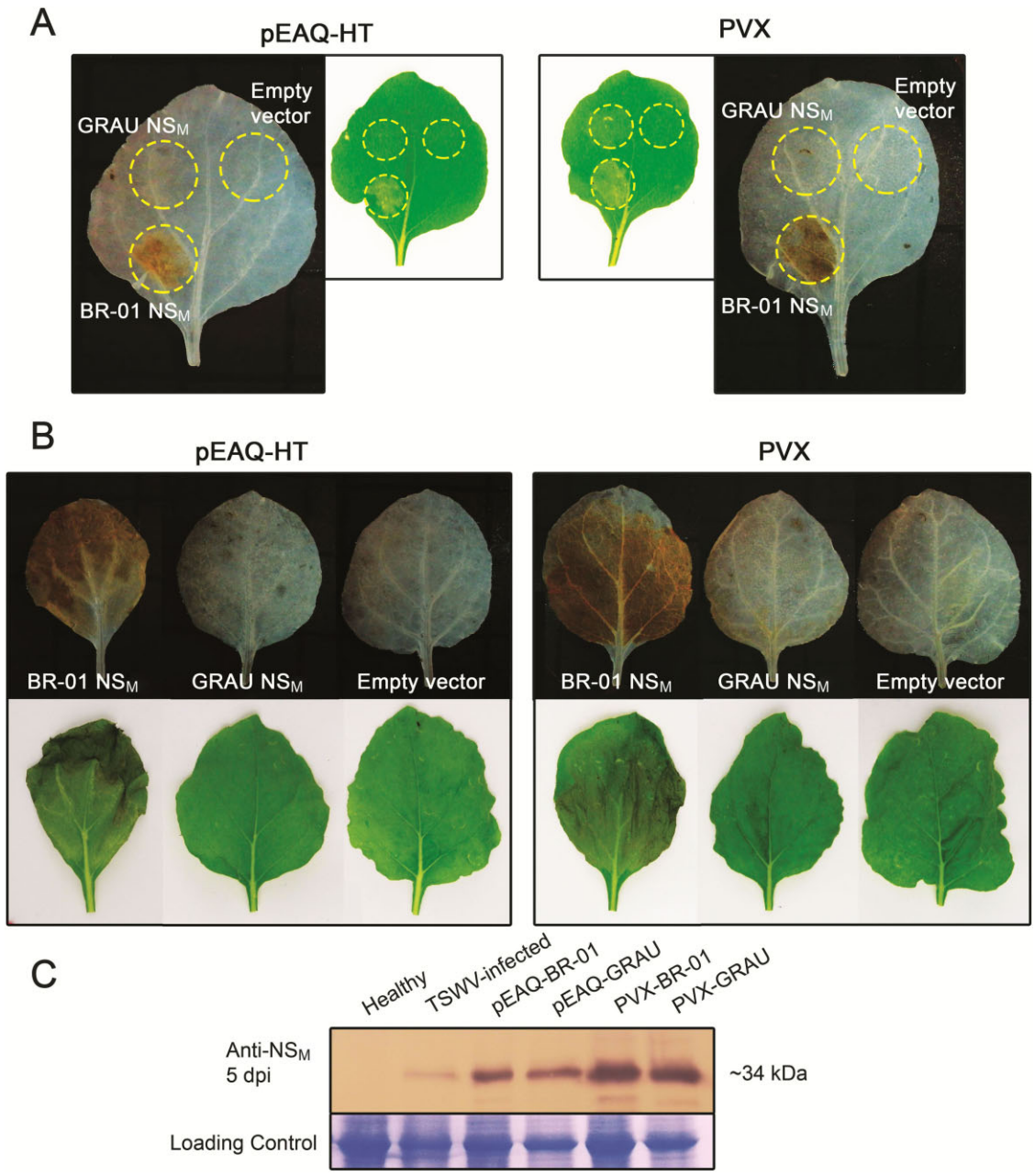

Fig. 3 Response of transgenic *Nicotiana benthamiana* (*Sw-5b*) leaves infiltrated with pEAQ-HT and *Potato virus X* (PVX) constructs expressing NS_M from isolates BR-01 and GRAU, respectively. Photographs of leaves shown in (A) and (B) were taken 5–6 days post-inoculation (dpi). In both panels, leaves were submitted to treatment with ethanol (destaining) for removal of chlorophyll to facilitate hypersensitive response (HR) visualization. (A) Individual leaf simultaneously infiltrated with three constructs pEAQ-HT-$NS_{M\text{-}BR\text{-}01}$, pEAQ-HT-$NS_{M\text{-}GRAU}$ and pEAQ-HT empty vector (left side) and with *Potato virus X* (PVX)-Gw-$NS_{M\text{-}BR\text{-}01}$, PVX-Gw-$NS_{M\text{-}GRAU}$ and PVX-Gw empty vector (right side), showing the visual response on infiltration. (B) Whole leaves infiltrated with pEAQ-HT-$NS_{M\text{-}BR\text{-}01}$, pEAQ-HT-$NS_{M\text{-}GRAU}$ and pEAQ-HT empty vector (left side) and with PVX-Gw-$NS_{M\text{-}BR\text{-}01}$, PVX-Gw-$NS_{M\text{-}GRAU}$ and PVX-Gw empty vector (right side), showing the visual response on infiltration. (C) Western immunoblot analysis with specific polyclonal antiserum against NS_M protein (34 kDa) of *Tomato spotted wilt virus* (TWSV). 34 kDa, molecular weight marker protein. Samples were taken from *N. benthamiana* (*Sw*-5b) plants inoculated with the wild-type TSWV virus and infiltrated with the constructs pEAQ-HT and PVX-Gw containing NS_M BR-01 and NS_M GRAU. Extract from healthy plants was used as a negative control.

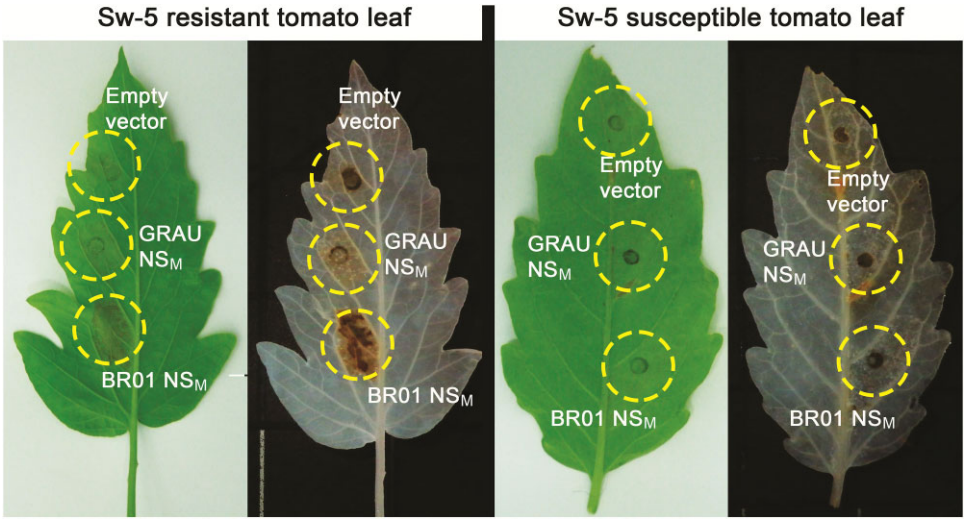

Fig. 4 Response of susceptible and resistant (harbouring the *Sw*-5 gene) tomato near-isogenic line leaves infiltrated with individual pEAQ-HT constructs expressing NS_M from isolate BR-01 or GRAU. Photographs of leaves shown were taken 4 days post-inoculation (dpi). In both cases, leaves were submitted to treatment with ethanol (destaining) for removal of chlorophyll to facilitate hypersensitive response (HR) visualization. Individual leaves from the resistant line (left side) and susceptible isoline (right side) were simultaneously infected with three constructs pEAQ-$HTNS_{M\text{-}BR\text{-}01}$, pEAQ-HT-$NS_{M\text{-}GRAU}$ and pEAQ-HT empty vector. Leaves of the resistant line showed the visual necrotic response (HR lesions) on infiltration.

Nowadays, RNA silencing is generally accepted as a virus-triggered immunity mechanism in plants and is suppressed by viral RNA silencing suppressor proteins, also referred to as effectors. *R*-gene-mediated immunity is a second line of defence that is triggered by effectors. The resulting arms race, nicely illustrated by the zig–zag model (Dangl and Jones, 2001), thereby implies a link between RNAi and *R*-gene-mediated immunity for viral pathogens with a key role for viral RNA silencing suppressor proteins as effectors. Having identified the NS_M movement protein as the *Avr* determinant for *Sw-5b* raises the question as to how this protein should be projected as an effector in this same zig–zag model. Only a few cases have been reported in which a plant viral cell-to-cell movement protein has been identified as an effector and has been simultaneously shown to modulate the antiviral RNAi response (Lanfermeijer *et al*., 2003; Vogler *et al*., 2008). Whether TSWV NS_M also contains such modulating activities remains to be investigated. Previously, TSWV NS_M has been reported to provoke the deposition of 1,3-β-D-glucan (GLU) or callose in mesophyll plasmodesmata (Pd), resembling the GLU depositions observed during an HR on viral infections (Rinne *et al*., 2005).

A recent bioinformatics analysis (López *et al*., 2011) on NS_M sequences available from databases indicated that a few amino acid substitutions are responsible for the breakdown of resistance in tomato lines carrying the *Sw-5* gene. Although no experimental data were provided in support, the authors suggested that the ability of TSWV to infect resistant plants was related to the replacement of a cysteine (C) with tyrosine (Y) at position 118 (C118Y) or a threonine (T) with asparagine (N) at position 120 (T120N). The RB isolate TSWV GRAU used in this study contained C and N at positions 118 and 120, respectively, whereas the RI isolate TSWV BR-01 contained C and T at these positions. Although TCSV and GRSV are classified as distinct tospovirus species, they are closely related to TSWV and are also able to induce the *Sw-5* resistance gene. Their NS_M amino acid sequences share ~85% similarity with TSWV NS_M from the RI isolate, and contain C and N residues at similar positions. Whether a change in these residues is indeed responsible (only) for overcoming the resistance conferred by the dominant *Sw-5* gene remains to be investigated. In the light of this, it is interesting to note that INSV also triggers *Sw-5* resistance and its NS_M (~68% identity to TSWV NS_M) contains residues H and T at amino acid positions 118 and 120, whereas MYSV does not trigger *Sw-5* resistance (sharing ~40% identity) and contains residues I and T at these positions (Fig. S3, see Supporting Information).

Although the *Sw-5b* gene was cloned a decade ago (Folkertsma *et al*., 1999; Spassova *et al*., 2001) and the *Avr* determinant of *Sw-5b*-governed resistance was mapped to the M RNA (Hoffmann *et al*., 2001), we have provided evidence that identifies NS_M as the trigger for *Sw-5* resistance. Unravelling the mechanism by which *Sw-5b* is triggered will now become a challenge and will not only contribute to provide an insight into dominant resistance genes, but may also help us to understand the broad-spectrum resistance of *Sw-5b* (against TSWV, TCSV, GRSV and INSV), a feature that is quite uncommon for a single dominant resistance gene, and which will be essential for the development of broad-spectrum resistance strategies.

EXPERIMENTAL PROCEDURES

Virus and plant material

Two TSWV isolates were used in this study, i.e. the *Sw-5b* RI reference strain BR-01 (de Ávila *et al*., 1993) and the RB isolate GRAU (Aramburu and Marti, 2003). Isolates were maintained by mechanical inoculations on *N. benthamiana* and grown under glasshouse conditions (24 °C with a 16-h light/8-h dark regime).

Nicotiana benthamiana transformation (*Sw-5b* gene)

The construct pBIN+Sw-5b containing the functional resistance gene copy *Sw-5b* was introduced into *A. tumefaciens* strain LBA4404 and used to generate *N. benthamiana* transformants, in a similar manner to the procedure described previously to generate transgenic *N. tabacum* SR1 containing various *Sw*-5 gene copies (Spassova *et al*., 2001).

Development of near-isogenic tomato lines harbouring the *Sw-5* gene

The *S. lycopersicum* cultivar 'Santa Clara' (highly susceptible to *Tospovirus* species) was crossed (as female parent) with the resistant cultivar 'Viradoro', a germplasm source of the *Sw*-5 locus derived from the tomato cultivar 'Stevens' (Dianese *et al*., 2011). After seven generations of backcross breeding (using 'Santa Clara' as a recurrent parent), a *Tospovirus*-resistant near-isogenic line was selected from a segregating F_8 population. This inbred line (named 'CNPH LAM 147') was phenotypically very similar to 'Santa Clara', differing, however, in its resistance response to *Tospovirus*, because of the presence of the genomic segment encompassing the locus with the *Sw*-5 gene cluster (Dianese *et al*., 2010). Progeny tests were carried out with 'Santa Clara' and 'CNPH LAM 147' in order to confirm the homozygous condition of both inbred lines. Seeds of these two genotypes were sown in 5-L pots filled with sterile soil and maintained in a glasshouse. Mechanical inoculation with TSWV (BR-01 isolate) was performed in 20 plants of 'Santa Clara' and 20 plants of the 'CNPH LAM 147' line, following standard procedures (Boiteux and Giordano, 1993). A co-dominant molecular marker system (Dianese *et al*., 2010), able to discriminate between heterozygous resistant (*Sw*-5/*sw*-5) and homozygous resistant (*Sw*-5/*Sw*-5) plants, was employed in conjunction with the inoculation assays, aiming to obtain two pure and contrasting near-isogenic lines. Total genomic DNA was extracted from asymptomatic 'CNPH LAM 147' and also from the mock-inoculated 'Santa Clara' plants. A single dominant homozygous resistant (*Sw*-5/*Sw*-5) plant of the line 'CNPH LAM 147' and a single recessive homozygous susceptible (*sw*-5/*sw*-5) 'Santa Clara' plant were selected. Seeds of these two contrasting genotypes were multiplied under glasshouse conditions and used in all subsequent assays.

Cloning of N, NS_M, NS_S and the GP genes into binary vectors

The TSWV BR-01 GP gene was excised with *Bam*HI from pBIN19-GP (Bucher *et al*., 2003) and, after agarose gel electrophoresis, purified and treated with T4 DNA polymerase. The pENTR11 entry vector was digested with *Eco*RI and, after agarose gel electrophoresis, was purified and religated to remove the *ccdB* region. The resulting pENTR11 vector, lacking the *ccdB* region, was digested with *Eco*RV and, after dephosphorylation, was used for cloning of the blunt-ended GP gene, yielding the vector pENTR11+GP_{BR-01}. The TSWV BR-01 NS_M gene was amplified by PCR from pGR107+$NS_{M\text{-}BR\text{-}01}$ (Lovato *et al*., 2008), and the GRAU NS_M gene was amplified by RT-PCR from total RNA of TSWV GRAU-infected *N. benthamiana* extracted with Trizol (Invitrogen, Carlsbad, CA, USA). For the cDNA synthesis, M-MLV reverse transcriptase was used (Promega, Madison, WI, USA). The PCRs were performed with primers (listed below) containing attB sites (Gateway Technology, Invitrogen), and the resulting PCR products were recombined into pDONR207 entry vector using BP Clonase (Invitrogen), yielding pDONR207+$NS_{M\text{-}BR\text{-}01}$ and pDONR207+$NS_{M\text{-}GRAU}$. Primers for BR-01 and GRAU NS_M amplification: NS_M-BR01-pD1 (GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGT TGACTCTTTTCGGTAACAA), NS_M-BR01-pD2 (GGGGACCACTTTGTACAAG AAAGCTGGGTCCTATATTTCATCAAAGGATAACTG), NS_M-GRAU-pD1 (GGG GACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTTGAC TTTTTTCAGCAATAAG) and NS_M-GRAU-pD2 (GGGGACCACTTTGTACAAGA AAGCTGGGTCCTATATTTCATCAAAAGATA ACTG).

The entry vectors pENTR11+GP_{BR-01}, pDONR207+$NS_{M\text{-}BR\text{-}01}$ and pDONR207+$NS_{M\text{-}GRAU}$ were recombined into Gateway-compatible pEAQ-HT and pGR107 (Lacorte *et al*., 2010) by LR Clonase (Invitrogen), yielding pEAQ-HT-GP_{BR-01}, pEAQ-HT-$NS_{M\text{-}BR01}$, pEAQ-HT-$NS_{M\text{-}GRAU}$, pGR107-$NS_{M\text{-}BR01}$ and pGR107-$NS_{M\text{-}GRAU}$. The binary vectors pEAQ-HT harbouring TSWV BR-01 N and NS_S genes were previously built and used as described (Ronde *et al*., 2013). All constructs were transformed into *A. tumefaciens* strain Cor308 or LBA4044. All standard procedures were performed following the manufacturers' recommendations and Green and Sambrook (2012).

Agroinfiltration and monitoring of HR

Agrobacterium infiltration assays were performed according to the protocol of Bucher *et al*. (2003), with slight modifications. To this end, *A. tumefaciens* Cor308 and LBA4044 Ooms *et al*. (1982), harbouring single constructs, were grown overnight at 28 °C in LB3 medium containing 2 μg/mL tetracycline and 100 μg/mL kanamycin selection pressure. From this culture, 600 μL were freshly inoculated into 3 mL of induction medium and grown overnight. Leaves were agroinfiltrated with a suspension containing a final optical density at 600 nm ($OD_{600\ nm}$) of 1.0 per construct. The development of HR was monitored daily up to 12 days after agroinfiltration. For ease of monitoring and confirmation of HR, chlorophyll was removed from infiltrated leaves with ethanol and acetic acid (3:1, v/v).

RNA extraction and RT-PCR

RNA extraction from wild-type and *Sw-5b*-transformed *N. benthamiana* leaves was carried out using Trizol reagent (Invitrogen). Amplification of N genes via RT-PCR was performed using M-MLV reverse transcriptase (Promega) and Go Taq DNA polymerase (Promega) with the specific primers: TSWV-N-F (ATGTCTAAGGTTAAGCTCACTA), TSWV-N-R (TCAAG CAAGTTCTGCGAGTTTT), INSV-N-F (ATGAACAAAGCAAAGATTACCA), INSV-N-R (TTAAATAGAATCATTTTTCCCA), MYSV-N-F (ATGTCTACCGTTGCT

AAGCTGA) and MYSV-N-R (TTAAACTTCAATGGACTT AGAT). All procedures followed the manufacturers' instructions.

Western immunoblot detection of viral proteins

For Western immunoblot detection of TSWV proteins, four leaf discs from *N. benthamiana* leaves were macerated in 400 μL of phosphate buffer (0.01 M, pH 7.0). The supernatant was collected and supplemented with 2 × Laemmli buffer (Sigma-Aldrich, St. Louis, MO, USA). Denatured proteins were resolved on 12.5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and, after electrophoresis, were transferred to Immobilon-P membrane (Millipore, Billerica, MA, USA). Blotted membranes were blocked with PBS (50 mM sodium phosphate buffer; 10 mM NaCl) containing 2% non-fat milk; the blots were then incubated using polyclonal antibodies (1 μg/mL) specific for TSWV N, NS_S, NS_M and GP proteins (de Avila *et al*., 1990; Kikkert *et al*., 1999; Kormelink *et al*., 1991, 1994).

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SUPPORTING INFORMATION

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

Fig. S1 Response of *Sw-5b* transgenic *Nicotiana benthamiana* on challenge with *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Melon yellow spot virus* (MYSV). Although TSWV and INSV induce a clear local hypersensitive response (HR), MYSV inoculation leads to a local and systemic infection.

Fig. S2 Response of untransformed, wild-type *Nicotiana benthamiana* leaves infiltrated with pEAQ-HT and *Potato virus X* (PVX) constructs expressing cell-to-cell movement protein (NS_M) from isolates BR-01 and GRAU.

Fig. S3 Alignment of the cell-to-cell movement proteins (NS_M) discussed in this study. Identical amino acid residues are shaded in grey. The arrow indicates position 120 for *Tomato spotted wilt virus* (TSWV) NS_M. GenBank accession numbers of the sequences: S58512 (TSWV BR-01), FM163370 (TSWV GRAU), AF513220 (*Groundnut ringspot virus*, GRSV), AF213674 (*Tomato chlorotic spot virus*, TCSV), M74904 (*Impatiens necrotic spot virus*, INSV) and AB061773 (*Melon yellow spot virus*, MYSV). The alignment was performed via Vector NTI (Invitrogen).