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The movement protein (NSm) of *Tomato spotted wilt virus* is the avirulence determinant in the tomato *Sw-5* gene-based resistance

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ABSTRACT

The avirulence determinant triggering the resistance conferred by the tomato gene *Sw-5* against *Tomato spotted wilt virus* (TSWV) is still unresolved. Sequence comparison showed two substitutions (C118Y or T120N) in the movement protein NSm present only in TSWV resistance-breaking (RB) isolates. In this work, transient expression of NSm of three TSWV isolates: RB1 (T120N), RB2 (C118Y) and non-resistance-breaking (NRB) in *Nicotiana benthamiana* expressing *Sw-5* showed only hypersensitive response (HR) with NRB. Exchange of the movement protein of *Alfalfa mosaic virus* (AMV) by the NSm supported cell-to-cell and systemic transport of the chimeric AMV RNAs in *N. tabacum* with or without *Sw-5* except for the constructs with NBR when *Sw-5* was expressed, although RB2 showed reduced cell-to-cell transport. Mutational analysis revealed that N120 is sufficient to avoid the HR but the substitution V130I was required for systemic transport. Finally, coinoculation of RB and NRB AMV chimeric constructs showed different prevalence of RB or NBR depending on the presence or absence of *Sw-5*. All results indicate that NSm is the avirulence determinant for *Sw-5* resistance and mutations C118Y or T120N are responsible for resistance-breakdown and have a fitness penalty in the context of the heterologous AMV system.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type member of the plant-infecting *Tospovirus* genus in the family *Bunyaviridae* (Milne and Francki 1984). The viral genome organization consists of three single-stranded RNAs: the large (L) negative sense RNA and the middle (M) and small (S) ambisense RNAs. Segment L (8.9kb) encodes an RNA-dependent RNA-polymerase (RdRp) (de Haan *et al.*, 1991); segment M (4.8kb) expresses from viral-sense (v) RNA the NSm which operates as a movement protein (MP) (Lewandowski and Adkins, 2005; Li *et al.*, 2009; Storms *et al.*, 1995), and from viral-complementary (vc) sense the precursor of surface glycoproteins G_N/G_C containing determinants for thrips transmission (Sin *et al.*, 2005); and segment S (2.9kb) encodes a silencing suppressor NS_S (Takeda *et al.*, 2002), in the viral-sense and the nucleopcapsid protein (N) from viral-complementary sense, used for encapsidation of viral RNA and, according to recent studies, facilitating long-distance movement (Feng *et al.*, 2013; de Haan *et al.*, 1990).

The management of the disease caused by TSWV has been extremely difficult because of its broad host range and the resistance of the thrips vectors to insecticides (Boiteux and Giordano, 1993). The highest level of resistance to TSWV was obtained by the introgression of the dominant single resistance genes *Tsw* in pepper and *Sw-5* in tomato. These genes were derived from *Capsicum chinese* and *Solanum peruvianum*, respectively (Boiteux, 1995; Moury *et al.*, 1998; Stevens *et al.*, 1991). The resistance mediated by *Sw-5* follows the gene-for-gene relationship (Staskawicz *et al.*, 1995) by triggering the typical hypersensitive response (HR) around the TSWV infection foci limiting virus spread to

distal parts of the plant. The avirulence (Avr) protein targeted by the resistance *Sw-5* gene is unknown, to date. Previous works revealed that the *Sw-5* locus contains at least five paralogs (denoted *Sw-5a* to *Sw-5e*) but only the *Sw-5b* gene, was necessary and enough to confer resistance against TSWV (Spassova *et al.*, 2001). The *Sw-5b* gene encodes a protein of 1246 amino acids and it is classified as a member of the coiled-coil, nucleotide-binding-ARC and leucine-rich repeat group of resistance gene candidates (Meyers *et al.*, 1999).

Control strategies based on *Sw-5* gene are affected by the emergence of TSWV resistance-breaking (RB) isolates able to overcome the resistance which have been reported in Republic of South Africa (Thompson and vanZijl, 1995), Hawaii (Canady *et al.*, 2001; Gordillo *et al.*, 2008), Australia (Latham and Jones, 1998), Spain (Aramburu and Marti, 2003) and Italy (Ciuffo *et al.*, 2005; Zaccardelli *et al.*, 2008). The lack of a TSWV infectious clone has hampered the study of the molecular mechanisms associated with *Sw-5* RB isolates. Previous analysis based on a complete set of reassortants generated from infectious mixture of two isolates of TSWV showed that the M segment has a major role in overcoming the *Sw-5* resistance (Hoffmann *et al.*, 2001). Moreover, the comparative analysis of nucleotide and amino acid sequences of RNA M from RB and non-resistance-breaking (NRB) isolates, revealed that the capacity to overcome the *Sw-5* resistance was associated to the presence of a tyrosine or an asparagine at positions 118 (Y118) or 120 (N120) of the NSm protein, respectively (Lopez *et al.*, 2011).

In the present work, we have analyzed the role of the NSm protein in the resistance mediated by the *Sw-5* gene by i), transient expression of the protein in *Sw-5* resistant plants (tomato, *Nicotiana tabacum* and *N. benthamiana*), in absence of other TSWV components; and ii), using the heterologous viral system based on *Alfalfa mosaic virus* (AMV), that

allows the functional exchangeability of viral movement proteins (MPs) assigned to the "30K family" (Fajardo *et al.*, 2013; Melcher, 2000; Sánchez-Navarro *et al.*, 2006). The results indicate that the NSm is the Avr factor of the *Sw-5b* gene, in which the Y118 or N120 residues are crucial to overcome the hypersensitive response.

RESULTS

Transient expression of TSWV NSm protein in *Sw5-b* transgenic *N. benthamiana* plants

To assess the direct role of the NSm protein of TSWV in the resistance mediated by *Sw-5* gene, in absence of other viral components, we performed a transient expression of the NSm protein in resistant *Sw5-b* transgenic *N. benthamiana* and/or *N. tabacum* lines. Both transgenic lines contain the same expression cassette, allowing the constitutive expression of the Sw5-b protein (Spassova *et al*., 2001; kindly provided by Dr. M. Prins). For this purpose, three NSm genes derived from two *Sw5*-RB (GRAU and Llo2TL3) and one *Sw5*-NRB (Gr1NL2) TSWV isolates (Lopez *et al*., 2011) were used in the present study. Each NSm of the RB isolates is representative of one of the two amino acids proposed by López *et al*. (2011) to be associated with *Sw-5* resistance overcome. Thus, while the NRB Gr1NL2 NSm (here after named as NRB) contains a cysteine and a threonine at positions 118 (118C) and 120 (120T), respectively, the NSm proteins of the RB Llo2TL3 (here after named as RB2) and GRAU (here after named as RB1) contain a tyrosine at position 118 (118Y) or an asparagine at position 120 (120N), respectively (supplementary Figure 1). In a preliminary study we observed that the TSWV isolates

GRAU and Gr1NL2 reproduced the expected phenotypes in *Sw5-b N. bethamiana* plants (supplementary Table 1). In the case of the RB TSWV Llo2TL3 isolate and due to the lack of infectious tissue, we used an AMV hybrid containing the NSm RB2 gene (see below). This hybrid virus infected locally and systemically the *Sw5-b N. bethamiana* plants without inducing any necrotic response. Later on, the NSm genes were cloned in a binary plasmid, being fused to HA epitope at its C-terminus, and transiently expressed by *A. tumefaciens* in wild type *N. benthamiana* (Nb/wt) or *N. tabacum* (Nt/wt) plants. Western blot analysis revealed that the three NSm proteins accumulated in agroinfiltrated leaves when transiently expressed in either Nb/wt or Nt/wt leaf with an electrophoretic mobility of the expected 35 kDa (Fig. 1A). However, the protein accumulation in *N. tabacum* plants was considerably lower (5 to 10 times) when compared to *N. benthamiana* plants. When transient expression of these three NSm proteins was assayed in susceptible and resistant tomato cultivars carrying the *Sw-5* gene, no expression at all was detected neither for the three NSm proteins nor for the control construct that carries the green fluorescent protein (GFP) (data not shown). Therefore, to overcome this problem, the different constructs were transiently expressed in transgenic *N. benthamiana* or *N. tabacum* plants constitutively expressing the *Sw-5* gene (Nb/Sw5-b; Nt/Sw5-b). The clearest results were observed in *N. bethamiana* plants. As shown in Figure 1B 6 days post-agroinfiltration, only the construct that contains the NRB gene triggered the hypersensitive like response on the Nb/Sw5-b leaf (Fig. 1B, column 4). These results clearly pointed at the *NSm* gene as the only TSWV component required to trigger the hypersensitive response mediated by the *Sw-5* gene.

Cell-to-cell and systemic movement of the chimeric AMV constructs with TSWV NSm in P12 *Nicotiana tabacum* plants

We analyzed the role of the *NSm* gene in the resistance mediated by the *Sw-5* gene, but in a viral context. For this purpose and due to the lack of an infectious TSWV clone, we used the heterologous AMV model system, which has been demonstrated to allow the functional exchangeability for the local (Sánchez-Navarro *et al*., 2006) and systemic (Fajardo *et al*., 2013) transport of MPs assigned to the 30K family. First of all, we analyzed the capacity of the three NSm proteins (NRB, RB1 and RB2) to support the local and systemic transport of chimeric AMV. To do this, the *NSm* gene was exchanged with the corresponding AMV MP gene in the AMV RNA 3 wt (pAL3NcoP3) (van der Vossen *et al*. 1993) or in a RNA 3 derivative that expresses the GFP (pGFP/A255/CP) (Sánchez-Navarro *et al.*, 2001). In the chimeric constructs the heterologous NSm proteins were extended with the C-terminal 44 residues (A44) of the AMV MP, to allow a compatible interaction with the AMV coat protein (CP) (Sánchez-Navarro *et al*., 2006).

Cell-to-cell movement of the AMV RNA 3 hybrids was studied by inoculation of T7 transcripts generated from the pGFP/NRB:A44/CP, pGFP/RB1:A44/CP and pGFP/RB2:A44/CP plasmids into transgenic *N. tabacum* plants that express constitutively the P1 and P2 polymerase proteins of AMV (P12) (Fig. 2A). All constructs resulted in clear fluorescent infection foci at 2 dpi (Fig. 2A) indicating that the three NSm proteins were competent to support the local transport of the hybrid AMV RNA 3. However, the analysis of the area of fifty independent foci at 2 and 3 dpi revealed that the foci derived from the pGFP/RB2:A44/CP construct were significantly smaller than those generated by pGFP/NRB:A44/CP and pGFP/RB1:A44/CP constructs (Student t-test, $p<0.05$) (Fig. 2B). Analysis of the replication of the constructs on P12 protoplast (Fig. 2C) did not suggest

significant RNA accumulation levels differences that could account for differences observed in the cell-to-cell movement.

The capacity of the different TSWV MPs to support the systemic transport of the AMV RNA 3 also was analyzed. For this purpose, we used the wild-type AMV RNA 3 constructs since the RNA 3 derivatives carrying the GFP reporter gene do not support systemic movement in P12 tobacco plants (Sánchez-Navarro *et al*., 2001). First, we observed that the different AMV RNA 3 hybrids accumulated comparable levels of RNAs 3 and 4 in P12 protoplast (Fig. 2D). The accumulation and distribution of the chimeric RNA 3s were then analyzed in inoculated and upper not inoculated leaves of P12 plants by tissue printing of petiole cross sections, in which positive hybridization signal always correlated with the presence of the virus in the corresponding leaf, as described previously (Fajardo *et al*., 2013; Mas and Pallás, 1995; Sánchez-Navarro *et al*., 2010). Results showed that, despite the differences observed in local movement, all AMV RNA 3 constructs were able to support systemic movement, infecting all upper leaves of P12 plants (Fig. 2E).

Analysis of the capability of the different AMV derivatives to overcome the resistance conferred by *Sw-5* in tomato and transgenic *N. tabacum* plants

In the next step we analyzed the capacity of the hybrid AMV to infect *Sw-5* resistant (Cultivar 'Verdi'; lanes 1 in Fig. 3) or TSWV-susceptible ('Marmande'; lanes 2 in Fig. 3) tomato cultivars. Therefore, the tomato plants were inoculated with wt AMV RNA 1 and RNA 2, purified CP and wt or chimeric RNA 3 constructs. Northern blot analysis of the inoculated tomato leaves in Fig. 3 shows the accumulation of the RNA 4, derived from the corresponding viral RNA 3. Similar accumulation levels were observed in the resistant or

susceptible tomato cultivars tested when the plants were inoculated with the AMV wt (Fig. 3, AMV RNA 4 band intensities: 40.7% vs. 35.3%, respectively), indicating that the genetic differences between the two tomato cultivars do not affect significantly the virus accumulation. A high accumulation level was observed in the susceptible tomato cultivar (Fig. 3, lanes 2) when inoculated with the chimeric AMV RNA 3 expressing the NRB protein (100%) followed by the chimeric AMV RNA 3 expressing the RB1 (56.6%) and RB2 (21.0%) proteins. These results indicated that in the tomato lacking the *Sw5-b* resistance gene the NRB NSm protein gave some advantages if compared to the RB NSm proteins or even to the wild type AMV MP. In the same tomato cultivar, it is remarkable the low accumulation level observed with the hybrid RNA 3 expressing the RB2 protein, whose sequence differs only in two or three residues compared to the RB1 or NRB proteins, respectively (see Supplementary figure 1). However, in the *Sw-5* resistant tomato cultivar (Fig. 3, lane 1) the presence of the NRB gene resulted into a significantly reduced (93%) accumulation (6.2% vs. 100%) whereas such a reduction was only at 5% (51.6% vs. 56.5%) or 9% (10.6% vs. 21.0%) for the AMV RNA 3 variants carrying the RB1 or RB2 genes, respectively. These results support that the presence of the NRB NSm protein, negatively affected the AMV accumulation in the *Sw-5* resistant tomato cultivar.

The presence of viral RNAs in upper non-inoculated leaves of the tomato cultivars was analyzed at 14 and 21 dpi by tissue printing analysis. No hybridization signal was detected in any of the plants analyzed, including those inoculated with the wt AMV, indicating that the AMV variant that we used to perform the analysis is unable to move systemically in tomato. To circumvent this limitation we used *N. tabacum* plants, which supported AMV local and systemic accumulation (see above). We then tested the chimeric

AMV constructs in both transgenic *N. tabacum* plants that express constitutively the *Sw5-b* gene (Nt/Sw5-b) (Spassova *et al*., 2001) and wild type *N. tabacum* plants (Nt/wt). Nt/wt and Nt/Sw5-b plants were inoculated as described above. The accumulation of the viral RNA on inoculated leaves was analyzed by Northern-blot at 7 dpi (Fig. 4). All AMV RNA 3 derivatives supported comparable levels of viral RNA 3 and 4 accumulations in Nt/wt and Nt/Sw5-b plants, except for the construct containing the NRB gene in Nt/Sw5-b plants, which accumulated 65% less efficiently (Fig. 4, lane 3). These results were equivalent to those obtained in resistant tomato plants (see above). We analyzed also the capacity of the heterologous MPs (NSm) to support the systemic transport of AMV RNA 3 by tissue printing (Sánchez-Navarro *et al*., 2010). Analysis of all upper not inoculated leaves of the Nt/wt and Nt/Sw5-b plants at 14 dpi revealed that all constructs rendered positive hybridization signal in both hosts, except the NRB:A44/CP RNA 3 hybrid which was exclusively detected in the susceptible Nt/wt plants (data not shown). To further confirm the accumulation of viral RNAs in the upper leaves, we performed a Northern-blot analysis of total RNA extracted from a mixture of the upper leaves U1, U2 and U3 (Fig. 4). The results showed that the three AMV RNA 3 chimeric variants NRB:A44/CP, RB1:A44/CP and RB2:A44/CP accumulated comparable levels of RNA 3 and 4 in the upper leaves of Nt/wt plants (Fig. 4, wt/systemic) indicating that the three NSm proteins are competent to support the systemic transport of viral RNAs. However, only the two RNA 3 constructs RB1:A44/CP and RB2:A44/CP were detected in the upper leaves of the resistant Nt/Sw5-b plants (Fig. 4, Sw5-b/systemic). This indicated that the NSm is the Avr determinant responsible to overcome the resistance mediated by the *Sw5-b* gene in the AMV viral context.

Mutational analysis of the RB and NRB NSm proteins

The amino acid alignments among RB and NRB NSm proteins pointed to the idea that the capability of TSWV to overcome the resistance mediated by *Sw-5* might be exclusively due to single changes present at residues 118 (Y) or 120 (N) of the NSm protein (Lopez *et al*., 2011), which are representative of the RB2 or RB1 NSm isolates analyzed herein. We cannot exclude, however, that other residues might be also contributing. Therefore, to analyze this aspect, we performed a mutational analysis using the RB1 and NRB NSm proteins, which differ only in two residues (RB2 and NRB differ in three residues) at position 120 (N in RB1 or T in NRB) and 130 (I in RB1 or V in NRB). By directed mutagenesis, we synthesized two variants of the RNA 3 for the heterologous AMV model system shown above, pGFP/RB1:A44/CP and pRB1:A44/CP constructs in which the asparagine at position 120 was changed to a threonine (pGFP/RB1-T120:A44/CP and pRB1-T120:A44/CP) or the isoleucine at position 130 was changed to a valine (pGFP/RB1-V130:A44/CP and pRB1-V130:A44/CP). The analysis of the cell-to-cell movement of the chimeric mutants expressing the GFP in *N. tabacum* P12 plants, revealed that at 3 dpi the presence of a T at position 120 in the RB1 protein (RB1-T120) increased significantly the area of the foci meanwhile the presence of a V at position 130 (RB1-V130) resulted into the opposite effect (Student t-test, $p<0.05$) (Fig. 5A). Those differences were not due to changes in the replication capability since all constructs accumulated comparable levels of viral RNAs on P12 protoplast (Fig. 5B). The capacity of both RB1 mutants to overcome the resistance mediated by the *Sw5-b* gene was analyzed by inoculation of *N. tabacum* Nt/wt and Nt/Sw5-b plants with transcripts derived from the two pRB1-T120:A44/CP and pRB1-V130:A44/CP mutant constructs, using pRB1:A44/CP

construct as control (Fig. 5C). Northern blot analysis of the inoculated and upper not inoculated leaves of Nt/wt plants revealed that the three constructs accumulated comparable levels of viral RNAs 3 and 4, indicating that none of the two changes introduced in the RB1 gene affected the capacity of the NSm protein to support the local and/or systemic transport of viral progeny. However, when the same analysis was performed with Nt/Sw5-b plants we observed that the three constructs were competent to infect the inoculated leaves, as shown by the accumulation of the viral RNAs 3 and 4, but only the construct containing the RB1 gene was detected in the upper not inoculated leaves. In addition, we observed differences in the symptomatology on the inoculated leaves of the resistant Nt/Sw5-b plants. Thus, while RB1 and RB1-V130 resulted in similar chlorotic spots, the construct carrying the RB1-T120 reproduced the typical necrotic lesions observed for the construct that expresses the NRB protein. Similar results were observed when the three NSm proteins (RB1, RB1-T120 or RB1-V130) were transiently expressed in Nb/Sw-5b plants in which only the RB1-T120 triggered the hypersensitive like response (Fig. 5 E, panel 3). All together, these results proved that mutations at position 120 are responsible for evading the hypersensitive response mediate by Sw-5 but also that in the context of the AMV system, other changes are required to compensate the putative fitness cost associated to the incorporation of the critical residue.

Competition assays

The presence of the TSWV RB isolates was associated mainly to *Sw-5*-resistant tomato crops, with scarce or null presence of these isolates in susceptible crops. This observation could suggest a fitness cost for RB TSWV isolates. The results obtained with the AMV model system and the different NSm proteins could suggest a possible fitness

cost associated to RB1 and RB2 (e.g. the reduced RNA accumulation in tomato or cell-to-cell transport in *N. tabacum*). Although we can not rule out that these effects are due to the heterologous AMV system or perhaps that other TSWV components could compensate the putative fitness cost effects (see below), we analyzed the relative fitness of chimeric AMV constructs carrying RB and NRB NSm genes to see if the pressure of the *Sw-5* could be sufficient to select the RB NSm proteins. To do this, a competition assay between RB1, RB2 and NRB NSm chimeric constructs was conducted by co-inoculation of *N. tabacum* P12 and Sw5-b expressing (Nt/Sw5-b) plants with an infectious mixture containing equivalent transcripts amounts. After two serial passages at 7 day intervals using extracts of the inoculated leaves as inoculum, the prevalent isolate present in the inoculated infected tissue was determined by direct sequence of the RT-PCR amplicons encompassing the full-length NSm gene. The results obtained in three independent experiments revealed that in P12 plants, all sequenced NSm amplicons corresponded to the NRB isolate meanwhile in Nt/Sw5-b plants, all the sequences corresponded to the RB1 isolate. These results suggested a fitness cost for RB strains in absence of the *Sw-5* gene pressure, whereas in *Sw-5* resistant genotypes, the AMV hybrid carrying the RB1 gene prevailed. Also, it should be noted that in the latter case, only the hybrid RNA 3 containing the RB1 gene was detected, thus suggesting a better fitness provided by this NSm under Sw-5 pressure.

DISCUSSION

The present analysis was addressed to experimentally confirm previous data suggesting that the NSm protein is the Avr determinant of TSWV in the resistance

mediated by *Sw-5* gene (Lopez *et al*., 2011). The initial results obtained by transient expression of RB and NRB NSm proteins in transgenic *N. benthamiana* cultivars carrying the *Sw5-b* gene (Nb/Sw5-b) revealed a hypersensitive (HR)-like response only with the NRB NSm protein, thus indicating unequivocally that NSm is the Avr determinant for the resistance provided by *Sw-5* gene. However, we were not able to reproduce the typical necrotic reaction to TSWV infection associated to the resistance mediated by the *Sw-5* gene, indicating that other factors could be modulating such phenotypic response, e.g. a putative high protein accumulation in the infected cells or an enhanced effect due to other cell responses associated to the viral infection. The observation that the HR-like response was clearly developed in *N. benthamiana* (Nb/Sw5-b) plants but not in *N. tabacum* (Nt/Sw5-b) plants, a host that accumulates 5 to 10 times lower protein titer in transient expression, could support the idea of a minimal protein accumulation threshold required to trigger the typical HR.

Furthermore, the differences between the NRB and the RB1 NSm proteins are exclusively located at position 120 (T or N) and 130 (V or I) but only the former was previously suggested by López *et al*. (2011) as responsible for overcoming the *Sw-5* resistance and it is necessary and sufficient to trigger the necrotic response (see below). Here we demonstrated that two (RB1) or three (RB2) residues confer the capacity to overcome the *Sw-5* resistance. Based on the gene-for-gene model of disease resistance described by Flor (1971), the few amino acids changes observed in the RB NSm proteins will maintain the pathogenic function but no longer the participation in the recognition event with the host resistance factor (Fraser, 1990). In agreement with this, we demonstrated that the two RB1 and RB2 proteins are still competent for the local and

systemic viral transport in the AMV heterologous system. The observation that few changes are associated with the capacity of an Avr gene to overcome a host resistance is a common property for different viral proteins as the MP (Meshi *et al.*, 1989; Calder and Palukaitis, 1992), the RNA polymerase (Meshi *et al.*, 1988; Padgett and Beachy, 1993) or the CP (Saito *et al.*, 1987; Dawson *et al.*, 1988) of tobamoviruses or the NSs protein of TSWV (Margaria *et al*., 2007; de Ronde *et al.*, 2013).

Another aspect was to know how the critical residues required to overcome the *Sw-5* resistance affect the functionality of the NSm proteins. This aspect was studied by using the AMV model system. The absence of other TSWV components in the AMV system allowed us to correlate any effect on the viral transport with the different residues present in the NSm protein although we cannot discard that the observed effect could be specific of the heterologous AMV system. Taking this in consideration we observed that the three NSm proteins used in the analysis were competent to support local and systemic transport of AMV in *N. tabacum* plants. However, we observed that the cell-to-cell transport of the chimeric AMV RNA 3 expressing the RB2 protein was significantly affected, showing infection foci with a reduced area. The differences in the amino acid NSm sequences observed among RB2 and the RB1 or NRB proteins analyzed, are located at positions 118 (Y), 130 (I) and 188 (T). The Y118 and I130 are present in NSm proteins of other TSWV isolates but T188 is exclusive of RB2 and the P321 isolates (GenBank accession number 307572726). This observation opens the possibility that the T188 is affecting the transport capacity of NSm protein. Further research is needed to confirm this hypothesis.

The AMV hybrids carrying the NSm genes were used to inoculate different plant species containing the *Sw-5* gene. Thus, we observed that the presence of the NRB NSm

gene was always correlated with a significant reduction of the accumulation of viral RNAs in the inoculated leaves of tomato or *Nicotiana* species tested, carrying the resistance gene *Sw-5*. This phenotype was also correlated with the absence of systemic virus infection. This result reproduces the same phenotype observed for the TSWV wild-type in these resistant plants, in which the NRB isolates are able to infect the inoculated leaves but they have lost the capacity to move to the upper part of the plant. All together, the results obtained in the present work support that the NSm protein is the Avr determinant for the resistance mediated by the dominant gene *Sw-5*.

Here we also analyzed if the critical Y118 or N120 residues, proposed by López *et al*. (2011) to be responsible to overcome the *Sw-5* resistance, are sufficient to trigger this phenotype. To answer this question we performed a mutational analysis using the RB1 protein that differ only in two residues (N120 or I130) with the corresponding ones of the NRB protein used herein. The analysis revealed that the N120 was required to avoid the hypersensitive response associated to *Sw-5*-resistant plants but also that this residue negatively affected the cell-to-cell transport in the AMV heterologous system. The conservation of this amino acid in all members of the genus *Tospovirus*, except in the TSWV resistance-breaking isolates (Lopez *et al*., 2011), supports the functional importance (strong negative selection) of this amino acid residue. On the other hand, the I130 significantly increased the cell-to-cell transport but triggered the hypersensitive response in infected Nt/Sw5-b or transiently expressed Nb/Sw5-b plants. Interestingly, none of the two single mutants was able to infect systemically the Nt/Sw5-b plants. These results suggested that the change T120N, present in the RB1, induces a fitness cost in the local movement of the chimeric construct that was confirmed by competition experiments. However, with the

AMV experimental system used we cannot rule out the possibility that this fitness cost could be specific of the heterologous system or perhaps overcome through secondary mutations (Sanjuan *et al.*, 2004; Sanjuan *et al.*, 2005) located out of the NSm protein. Additionally, the change V130I, present in the NSm of most of the TSWV isolates available in databases (503 out 504 sequences), seems to be a positive selected residue for an efficient cell-to-cell viral movement. Our results suggest that the RB isolates will appear only in a I130 background. The fitness penalties is a prerequisite for both the resistance genes (R) and Arv genes in the different models proposed for the coevolution of the host-parasite in a gene-for-gene system (Sasaki, 2000; Bergelson *et al.*, 2001; Burdon and Thrall, 2003; Segarra, 2005). This assumption is also supported by the small size of virus genomes, in which any modification of the few encoded multifunctional proteins, could result in a fitness cost (Sacristan and Garcia-Arenal, 2008; Fraile and Garcia-Arenal, 2010). It was suggested that even a limited number of nucleotide changes in the virus genome may have strong pleiotropic effects. Mutations responsible for gains of virulence frequently induce fitness costs to the virus in plants which devoid of the corresponding resistance. This was shown in several instances (Goulden *et al.*, 1993; Jenner *et al.*, 2002; Desbiez *et al.*, 2003; Lanfermeijer *et al.*, 2003; Ayme *et al.*, 2006; Agudelo-Romero *et al.*, 2008) although it cannot be generalized because there are examples of virulent strains that are at least as fit as the avirulent ones (Sorho *et al.*, 2005; Chain *et al.*, 2007). High fitness penalties associated with increased pathogenicity has been inferred for different plant viruses from direct (Fraile *et al.*, 2011) or indirect evidences (Murant *et al.*, 1968; Hanada and Harrison, 1977; Culver *et al.*, 1994; Mestre *et al.*, 2003). The results presented herein supported a high fitness penalty associated to the RB NSm gene, at least in the AMV

system. This was confirmed experimentally by competition experiments in which the chimeric NRB RNA 3 outcompeted the RB1 and RB2 constructs in the absence of the *Sw-5* resistance gene, whereas the RB1 variant was the prevalent one in the Sw-5 resistant background even outcompeting the RB2. This latter result also suggested that the RB1 NSm isolate has less fitness penalty than the RB2, at least in the resistant genotype, an effect that could be the consequence of a more permissive amino acid changes or a more competitive evolved *NSm* gene. It is remarkable that most codons of the *NSm* were found to be under neutral or purifying selection and only a positive selection was detected at codon 118 due to the adaptation to overcome the resistance conferred be the *Sw-5* gene (Lopez *et al*., 2011). The same observation was suggested for the substitution T120N although the small number of isolates showing this change might have precluded its detection by the statistical methods used (Lopez *et al*., 2011). The results presented herein supported a positive selection for the N120 under the resistance gene *Sw-5* selection pressure. Additionally, the observation of different fitness penalties between the two RB NSm would indicate that both genes are evolving to compensate for the fitness loss associated to these amino acid changes (Y118 or N120). If this is the scenario, then the question will be how long it will take for other mutations to appear in RB NSm able to compete (with similar or higher fitness) the NRB NSm in a context in which absence of the resistance gene *Sw-5* occurs. Further research will be needed to study this aspect and to confirm if the results obtained with the AMV system could be applied to the TSWV.

EXPERIMENTAL PROCEDURES

Recombinant plasmids for introducing the NSm genes in the AMV RNA 3 and for its transient expression.

A modified infectious AMV cDNA 3 clone, which expresses the green fluorescent protein (GFP) (pGFP/A255/CP) (Sánchez-Navarro and Bol, 2001), was used to exchange the N-terminal 255 amino acids of the AMV MP gene with the corresponding MP gene (NSm) of *Tomato spotted wilt virus* (TSWV). Three TSWV isolates derived from natural infections of tomato, two *Sw-5*-RB named GRAU (GenBank FM163370) and Llo2TL3 (GenBank HM015518), and *Sw5*-NRB Gr1NL2 (Genbank HM015513) were used as templates to amplify the MP gene (Lopez *et al*., 2011) by using specific primers, The MP genes are referred hereafter as RB1 (GRAU isolate), RB2 (Llo2TL3 isolate) or NRB (Gr1NL2 isolate). The digested fragments were used to replace the *Nco*I–*Nhe*I fragment of pGFP/A255/CP, corresponding with the N-terminal 255 amino acids of the AMV MP, to generate the constructs: pGFP/RB1:A44/CP, pGFP/RB2:A44/CP and pGFP/NRB:A44/CP.

Additionally, the TSWV MP genes were introduced in an infectious cDNA 3 clone of AMV wt (pAL3NcoP3) (van der Vossen *et al.*, 1993) by exchanging the *Nco*I-*Pst*I fragment between the pAL3NcoP3 plasmid and the pGFP/A255/CP derivatives, described above. The resultant chimeric plasmids were referred as pRB1:A44/CP, pRB2:A44/CP and pNRB:A44/CP.

The pGFP/RB1:A44/CP and pRB1:A44/CP plasmids were used as templates to introduce by directed mutagenesis the substitution T120 (substitution N for T at position 120) and V130 (substitution I for V at position 130) of the MP, resulting the mutant constructs pGFP/RB1-T120:A44/CP or pGFP/RB1-V130:A44/CP and pRB1-T120:A44/CP or pRB1-V130:A44/CP, respectively.

For the transient expression of the different TSWV MPs, the previously amplified MP genes were introduced in the expression cassette of the plasmid pSK+ 35S–MPPNRSV:HA-PoPit (Martinez-Gil *et al.*, 2009), by exchanging the *Prunus necrotic ringspot virus* (PNRSV) MP gene. The resulting cassettes will contain the corresponding TSWV MP fused to the hemagglutinin (HA) epitope at its C terminus. Each cassette was introduced into the pMOG800 binary vector by using a unique *Xho*I restriction site.

Inoculation of *Nicotiana tabacum* plants and tomato cultivars.

pAL3NcoP3, pGFP/A255/CP and the corresponding NSm derivatives, were linearized with *Pst*I and transcribed with T7 RNA polymerase. The transcripts were inoculated onto transgenic *N. tabacum* plants that express constitutively the P1 and P2 polymerase proteins of AMV (P12), as previously described (Taschner *et al.*, 1991). The fluorescence derived from the chimeric AMV RNA 3 carrying the GFP, was monitored using a Leica Stereoscopic Microscope. The area of infection foci was measured at 2 and 3 days post-inoculation (dpi), using Image J software (Wayne, Rasband, National Institutes of Health, Bethesda, MD, USA; http://rsbweb.nih.gov/ij).

N. tabacum wild type plants (Nt/wt) or *N. tabacum* plants expressing constitutively the resistance gene *Sw5-b* (Nt/Sw5-b) (Spassova *et al.*, 2001) and the tomato cultivars, "Verdi" (heterozygous for the *Sw-5* resistance gene) and "Marmande", which do not carry *Sw-5* (provided by Semillas Fitó, Barcelona, Spain) were inoculated with a mixture of capped transcripts corresponding to AMV RNAs 1, 2, the wild type or chimeric RNA 3 plus a few micrograms of purified AMV CP as described (Neeleman and Bol, 1999).

For the competition assays, the inoculum contained a mixture of AMV RNAs 1 and 2 plus the three RNA 3 transcripts, at the same concentration, derived from the pRB1:A44/CP, pRB2:A44/CP and pNRB:A44/CP plasmids. P12 and Nt/Sw5-b plants were inoculated as described above and two serial passages at 7 dpi were performed using an extract of the inoculated leaves as inoculum.

Northern blot and Tissue printing assays.

Tissue printing analysis were performed using transversal section of the corresponding petiole, as described previously (Fajardo *et al*. 2013). Total RNA was extracted from inoculated (I) and upper (U) not inoculated leaves at 7 dpi and 14 dpi, as described previously (Sánchez-Navarro *et al.*, 1997). In the case of the upper leaves, the RNA extraction was performed using a mixture of U3, U4 and U5 leaves, in which U1 correspond to that closest to the inoculated leaf. Hybridization and detection was conducted as previously described (Pallás *et al*. 1998) using a dig-riboprobe (Roche Mannheim, Germany) complementary to the AMV 3' untranslated region (UTR). The intensity of the bands was quantified using the ImageJ 1.48c software (http://imagej.nih.gov/ij).

Transient expression of the TSWV MPs *in planta* and Western Blot assay.

Agrobacterium tumefaciens, strain C58, transformed with the corresponding binary pMOG 800 plasmids, were grown overnight in a shaking incubator at 28 ºC in Luria-Bertani (LB) medium supplemented with the appropriate antibiotic. Cultures were collected by centrifugation and adjusted to 0.5 optical density (OD600) with 10 mM $MgCl_2$, 10 mM MES pH 5.6 and 150 µM acetosyringone. These suspensions were used to infiltrate the different plants as described (Herranz *et al.*, 2005). The expression of the different viral

MPs was analyzed by Western blot assay as described (Martinez-Gil *et al*., 2009). Blots were developed using an ECL+ Plus Western Blotting Detection System (Amersham) and the LAS-3000 digital imaging system (FujiFilm). The intensity of the bands was quantified using the ImageGauge 4.0 software (FujiFilm).

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SUPPORTING INFORMATION LEGENS

Fig. S1 Sequence alignment of the three NSm proteins derived from the two TSWV resistance-breaking (RB) isolates GRAU (RB1; GenBank FM163370) and Llo2TL3 (RB2; GenBank HM015518) and the non-resistance-breaking (NRB) isolate Gr1NL2 (Genbank HM015513). The amino acids of the RB isolates that differs from the NRB variant, are indicate in red.

Table S1 Symptoms observed in *N. benthamiana* (Nb) or *N. tabacum* (Nt) plants wild type (wt) or carrying the Sw5-b gene (Sw5-b), inoculated with TSWV or chimeric AMV constructs.

FIGURE LEGENDS

Fig. 1 Transient expression of the *Tomato spotted wilt* (TSWV) NSm proteins (MP) in wild type *N. benthamiana* (Nb/wt) or *N. tabacum* (Nt/wt) and transgenic *N. benthamiana* plants carrying the resistance gene *Sw5-b* (Nb/Sw5-b). **A**, Western blot analysis of the Nb/wt and Nt/wt infiltrated leaves at 3 days post infiltration expressing RB1:HA (lane 1), RB2:HA (lane 2) and NRB:HA (lane 3). Lanes M and 4 correspond to non-agroinfiltrated leaves and leaves infiltrated with cultures carrying the empty binary plasmid, respectively. The numbers at the bottom of the panel represent the relative percentage of the intensity of each band with respect the more intense one in lane 1. **B**, Pictures of Nb/Sw5-b (upper part) or Nb/wt (lower part) leaves expressing RB1:HA (1), RB2:HA (2) or NRB:HA (3) at 6 days post agroinfiltration.

Fig. 2 Analysis of the accumulation, cell-to-cell and systemic transport of the *Alfalfa mosaic virus* (AMV) chimeric RNAs carrying the MP of *Tomato spotted wilt* (TSWV) isolates. **A**, Infection foci observed in P12 plants inoculated with RNA 3 transcripts from pGFP/A255/CP derivatives, which contain the TSWV NSm RB1 (2), RB2 (3) or NRB (3) genes. The schematic representation shows the GFP/A255/CP AMV RNA 3 (1), in which the open reading frames corresponding to the green fluorescent protein (GFP), the movement protein (MP) and the coat protein (CP) are represented by large boxes. The number showed in the MP box represents the total amino acids residues of the AMV MP (255) exchanged for the TSWV NSm, represented by single boxes below. The *Nco*I and *Nhe*I restriction sites used to exchange the MPs genes are indicated. The arrows indicate the subgenomic promoters. The C terminal 44 amino acids of the AMV MP are indicated as A44. Images correspond to representative pictures of the infection foci observed at 2 days post-inoculation (dpi) using a Leica Stereoscopic Microscope. Scale bar corresponds to 2 mm. **B**, Histograms represent the average of the area of 50 independent infection foci at 2 and 3 dpi developed in P12 plants inoculated with transcripts derived from the AMV RNA 3 variants shown in (A). Error bars indicate the standard deviation. **C**, Northern blot analysis of the accumulation of the chimeric AMV RNAs in P12 protoplasts inoculated with RNA transcribed from the constructs shown in (A). **D**, Northern blot analysis of the accumulation of the chimeric AMV RNAs lacking the 5' proximal GFP gene, in P12 protoplasts. P12 protoplasts were inoculated with RNA transcribed from plasmid pAL3NcoP3 derivatives, expressing the AMV MP (lane 1) or the NSm RB1 (lane 2; plasmid pRB1:A44/CP), RB2 (Lane 3, plasmid pRB2:A44/CP) and NRB (lane 4; plasmid pNRB:A44/CP). The position of the chimeric RNA 3 and 4 and additional subgenomic

RNA (sgRNA) are indicated on the left margin. **E**, Tissue printing analysis of P12 plants inoculated with the AMV RNA 3 derivatives used in (D). Plants were analyzed at 14 dpi by printing the transversal section of the corresponding petiole from inoculated (I) and upper (U) leaves. The position of each leaf is indicated by numbers which correspond to the position of the leaves in the plant from the lower to the upper part in which U1 corresponds to the closest one to the inoculated leaf. rRNA indicates 23S RNA loading control. M refers to mock inoculated plant.

Fig. 3 Northern blot analysis of the *Alfalfa mosaic virus* (AMV) chimeric RNAs accumulation in the inoculated leaves of *Sw-5* resistant "Verdi" (lanes 1) or *Tomato spotted wilt virus* (TSWV)-susceptible "Marmande" (lanes 2) tomato cultivars. The tomato plants were inoculated with the corresponding RNA 3 transcript expressing the AMV MP (AMV wt) or the NSm of the TSWV isolates Gr1NL2 (NRB), GRAU (RB1) and Llo2TL3 (RB2). Mock (M), represents total RNA extraction of healthy tissue. The position of the RNA 4 is indicated at the left margin of the picture. rRNA indicates 23S RNA loading control. The numbers below the panel represent the relative percentage of the intensity of each band with respect to the more intense one (lane 2/NRB).

Fig. 4 Northern blot analysis of the *Alfalfa mosaic virus* (AMV) chimeric RNAs accumulation in transgenic *Nicotiana tabacum* plants that express constitutively the *Sw5-b* gene (Nt/Sw5-b). *N. tabacum* wild type (Nt/wt) and Nt/Sw5-b plants were inoculated as described in Figure 3, in which the RNA 3 transcripts expresses the NSm RB1(lane 1), RB2 (lane 2) and NRB (lane 3). The analyzed RNAs from inoculated leaves corresponded to a

mixture of total RNA extracted from the two inoculated leaves (I1 and I2) at 7 dpi whereas the analyzed RNA from systemic leaves corresponded to a mixture of the total RNAs extracted from the upper (U) leaves U1, U2 and U3 at 14 dpi. The positions of the chimeric RNA 3 and RNA 4 are indicated on the left margin of the pictures. rRNA indicates 23S RNA loading control.

Fig. 5 Functional characterizations of NSm RB1 single mutants. **A**, Histograms represent the average of the area of 50 independent infection foci at 2 and 3 dpi observed in *Nicotiana tabacum* P12 plants inoculated with transcripts from *Alfalfa mosaic virus* (AMV) RNA 3 pGFP/A255/CP derivatives pGFP/RB1:A44/CP (lane 2), pGFP/RB1-T120:A44/CP (lane 3) and pGFP/RB1-V130:A44/CP (lane 4). The fluorescent infection foci were visualized using a Leica Stereoscopic Microscope. Error bars indicate the standard deviation. **B**, Northern blot analysis of the accumulation of chimeric AMV RNAs in P12 protoplasts inoculated with RNA transcripts derived from the constructs used in (A) plus the plasmid pGFP/A255/CP (lane 1). **C**, Northern blot analysis of the accumulation of the chimeric AMV RNAs in *N. tabacum* plants that express constitutively the Sw5-b gene (Nt/Sw5-b) or *N. tabacum* wild type (Nt/wt) plants. All plants were inoculated as described in Figure 3 in which the chimeric RNA 3 corresponds to the transcripts derived from the constructs pRB1:A44/CP (lane 2), pRB1-T120:A44/CP (lane 3) and pRB1-V130:A44/CP (lane 4). Total RNA was extracted from inoculated and upper leaves as described in Figure 4. The positions of the chimeric RNA 3 and RNA 4 are indicated at the left margin of the pictures. **D**, Symptomatology observed in Nt/Sw5-b plants inoculated with chimeric AMV derivatives used in (C) at 6 dpi. **E**, Pictures of Nb/Sw5-b leaves expressing TSWV NSm

RB1:HA (2), RB1-T120:HA (3) and RB1-V130:HA (4) at 6 days post agroinfiltration. Mock (M), represents total RNA extraction of healthy tissue. rRNA indicates 23S RNA loading control.

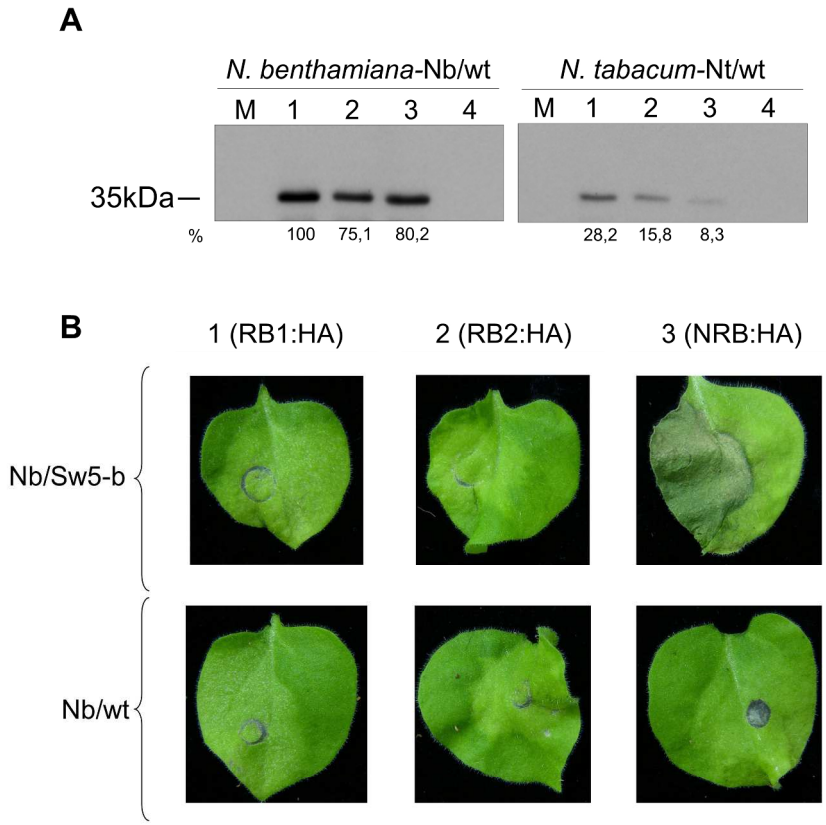

Figure 1
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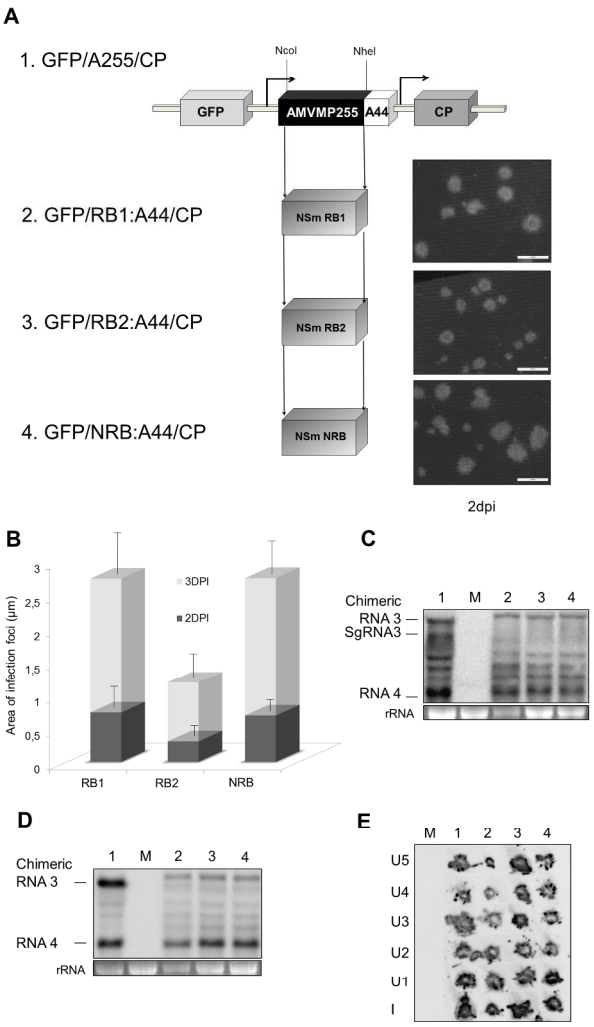

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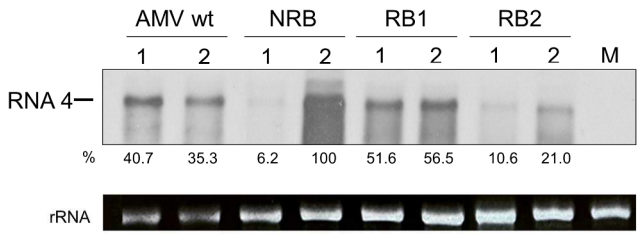

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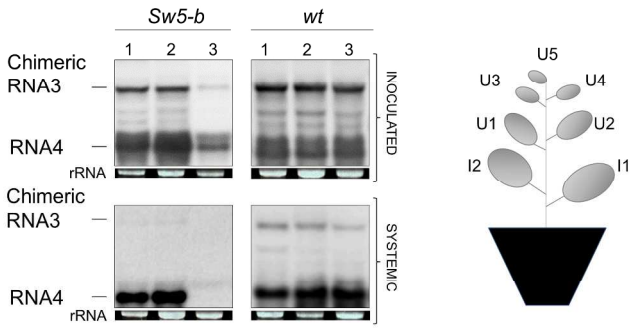

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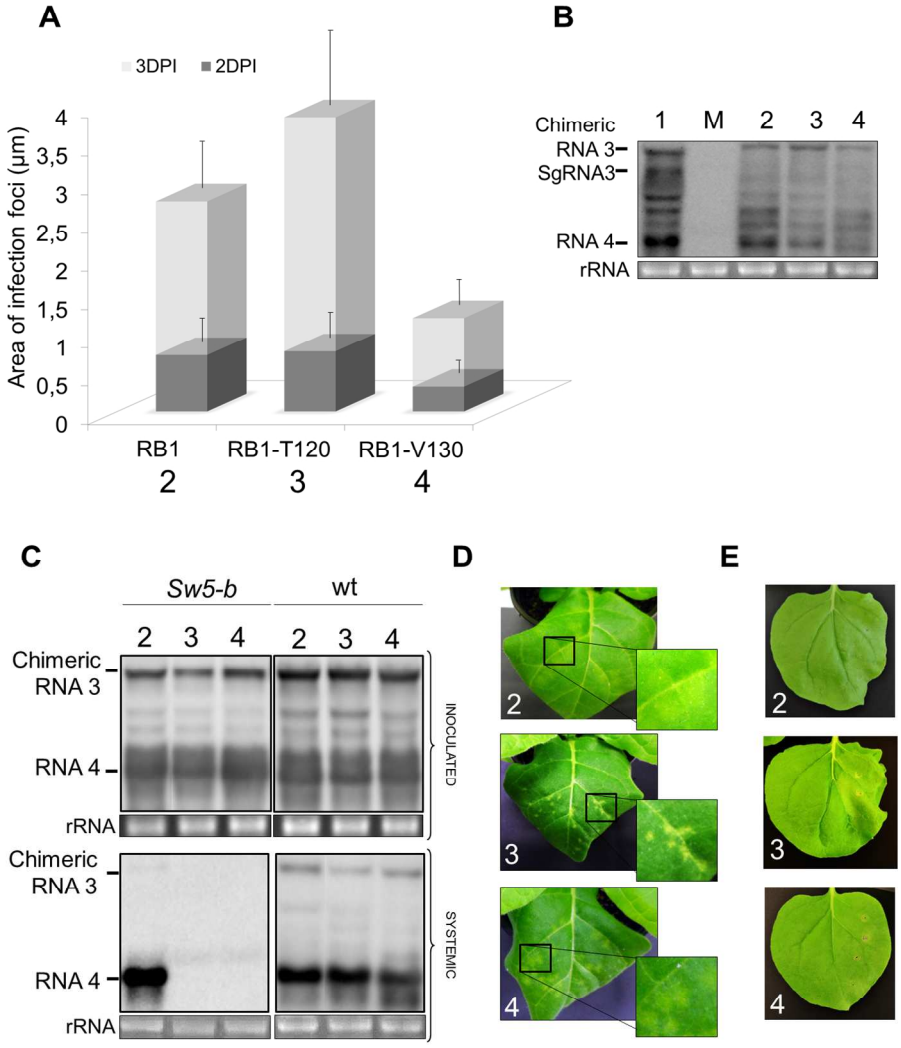

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