Plasmodesmata targeting and intercellular trafficking of Tomato spotted wilt tospovirus movement protein NSm is independent of its function in HR induction

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The movement protein NSm of Tomato spotted wilt tospovirus (TSWV) plays pivotal roles in viral intercellular trafficking. Recently, the TSWV NSm was also identified as an avirulence (Avr) determinant during the Sw-5b-mediated hypersensitive response (HR). However, whether the cell-to-cell movement of NSm is coupled to its function in HR induction remains obscure. Here, we showed that the NSm mutants defective in targeting plasmodesmata and cell-to-cell movement were still capable of inducing Sw-5b-mediated HR. In addition, introduction of a single amino-acid substitution, C118Y or T120N, identified previously from TSWV resistance-breaking isolates, into the movement-defective NSm mutants resulted in the failure of HR induction. Collectively, our results showed that the intercellular trafficking of NSm is uncoupled from its function in HR induction. These findings shed light on the evolutionary mechanism of R-Avr recognition and may be used to explain why this uncoupled phenomenon can be observed in many different viruses.

Tomato spotted wilt virus (TSWV) is the type species of Tospovirus, the only genus of plant-infecting viruses in the family Bunyaviridae (Elliott, 1990, 1996; Goldbach & Peters, 1996). TSWV causes severe economic losses in many agronomic and ornamental crops worldwide (Hanssen et al., 2010). The tomato Sw-5 is known to confer broad-spectrum resistance to tospoviruses including TSWV, Groundnut ring spot virus (GRSV) and Tomato chlorotic spot virus (TCSV) (Bendahmane et al., 2002; Boiteux & Giordano, 1993). This Sw-5b resistance gene has been cloned and shown to contain a coiled-coil, a central nucleotide-binding site (NBS), C-terminal leucine-rich repeats (LRRs), and an extra N-terminal domain (Brommonschenkel et al., 2000; Spassova et al., 2001). The avirulence (Avr) factor of the tomato Sw-5b-resistance protein has been determined as the nonstructural movement protein NSm of TSWV (Hallwass et al., 2014; Lopez et al., 2011; Peiró et al., 2014). Transient expression of the NSm protein in leaves of Sw-5 tomato or Sw-5 transgenic Nicotiana benthamiana resulted in a hypersensitive response (HR) (Hallwass et al., 2014; Peiró et al., 2014).

The NSm protein of TSWV has typical characteristics of plant viral movement protein, including localization into plasmodesmata (PD) (Feng et al., 2016; Kormelink et al., 1994), modification of size-exclusion limit of PD (Prins et al., 1997; Storms et al., 1998), tubule formation (Storms et al., 1995), RNA-binding activity and interaction with N protein (Soellick et al., 2000). Using a heterologous Tobacco mosaic virus (TMV)-expression system, domains and amino-acid residues of the TSWV NSm protein necessary for tubule formation, intercellular movement and symptom

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development were identified (Li et al., 2009). The NSm protein was also shown to play pivotal roles in cell-to-cell movement of viral ribonucleoproteins (RNPs) of TSWV or facilitate intercellular movement of heterologous viruses (Feng et al., 2016; Kormelink et al., 1994; Lewandowski & Adkins, 2005; Li et al., 2009). To date, whether the cell-to-cell movement function of NSm is coupled with its function in HR induction remains obscure.

The coding regions of TSWV NSm and tomato Sw–5b were amplified from the cDNA of TSWV-YN isolate (accession number JF960236.1) and the genomic DNA of tomato cultivar 43419, respectively, using PrimeStar HS DNA Polymerase (TaKaRa, Dalian, China). The resulting products were constructed, respectively, into a pCambia2300 vector with the phenotype shown on the leaves, H. benthamiana leaves also did not produce HR (Fig. 1a). 3,3'-Diaminobenzidine (DAB) staining showed a strong accumulation of H2O2 in the cells co-infected with NSm and Sw–5b by 24 h post infiltration (hpi, Fig. 1d). Consistent with the phenotype shown on the leaves, H2O2 accumulation was not detected in the leaf tissues infiltrated with Sw–5b, NSm or EV alone (Fig. 1b, c and e). These data indicated clearly that the HR can only be induced after co-infiltiration with NSm and Sw–5b.

PD targeting is known to be an important step during cell-to-cell movement of TSWV NSm (Feng et al., 2016; Kormelink et al., 1994) and many other viral movement proteins (Benitez-Alfonso et al., 2010; Lucas, 2006). In 2009, Li and others reported several mutants of TSWV NSm defective in tubule formation and cell-to-cell movement. To determine whether the cell-to-cell movement function of NSm is coupled with its function during Sw–5b-mediated HR, we decided to re-construct A54–56, A93–94, A122–125, A154 and A269–274 mutants (Li et al., 2009) and use them in our subsequent PD-targeting assays. The corresponding alanine substitutions were individually introduced into the NSm protein, fused with a yellow fluorescence protein at its C-terminus to generate NSmA54–56-YFP, NSmA93–94-YFP, NSmA122–125-YFP, NSmA154-YFP and NSmA269–274-YFP, respectively. After agro-infiltration of these constructs individually into N. benthamiana leaves, we examined their subcellular localization patterns under a CLSM. To visualize the PD in cell walls, we utilized a construct expressing the TMV movement protein (MP) fused with a red fluorescence protein at its C terminus (TMV MP-RFP). At 24 and 48 hpi, and under the confocal microscope, the TMV MP-RFP fusion was seen as red dots at the PDs in cell walls (Fig. 2b). When co-infiltrated TMV MP-RFP and individual NSm-YFP constructs into cells, the NSm-YFP fusion revealed a co-localization with the TMV MP-RFP fusion at PDs (Fig. 2a–c). In the cells co-infiltrated with TMV MP-RFP and NSmA54–56-YFP, NSmA93–94-YFP, NSmA122–125-YFP, NSmA154-YFP or NSmA269–274-YFP, no co-localization of the two fusion proteins at PD was observed, indicating that none of the mutant NSm proteins was able to target PD (Fig. 2d–r).

Li et al. (2009) performed cell-to-cell movement assays in leaves of Nicotiana tabacum var. Xanthi and N. benthamiana using TMVcpNSm RNA transcripts. In this study, the cell-to-cell movement of NSm mutants (NSmA54–56, NSmA93–94, NSmA122–125, NSmA154 and NSmA269–274) was assayed using a completely different system (Shen et al., 2014). All the mutant NSm sequences were placed behind a 2×35S promoter in a binary vector and lacked the YFP fusion. The wild-type TSWV NSm construct (TSWV NSm Wt) and the mutant constructs were agro-infiltrated into N. benthamiana leaves together with a CMV RNA3-MP::erGFP reporter described previously (Shen et al., 2014). A CMV-MP construct was used in this study as a positive control. As shown in Fig. 2s, expression of the CMV RNA3-MP::erGFP reporter alone in cell resulted in single cells showing green fluorescence. When CMV RNA3-MP::erGFP reporter was co-expressed with the CMV MP (Fig. 2t) or TSWV NSm (Fig. 2u) construct, the green-fluorescence signal from the CMV RNA3-MP::erGFP reporter was seen in many cells indicating that the reporter had moved efficiently from the originally infiltrated cells into the neighbouring cells. When the CMV RNA3-MP::erGFP reporter was co-infiltrated with individual mutant constructs produced in this study into N. benthamiana leaves, the green-fluorescence signal was only seen in single cells (Fig. 2v–z).
These results confirmed the results of Li and colleagues, using completely new constructs and cell-to-cell movement-assay system.

Next, we investigated whether these NSm mutants were still able to induce HR. Each NSm mutant was co-infiltrated with Sw-5b into leaves of *N. benthamiana*. As shown in Fig. 1f, all the NSm mutants (A54–56, A93–94, A122–125, A154 and A269–274) did elicit HR response in *N. benthamiana* leaves in the presence of Sw-5b. The HR responses induced by these mutants were indistinguishable from the HR response induced by the wild-type NSm. Immunoblot analysis confirmed the expression of all the mutants in the

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**Fig. 1.** NSm mutants defective in PD targeting or in cell-to-cell movement can trigger *Sw-5b*-mediated hypersensitive response (HR) in *Nicotiana benthamiana*. (a) *N. benthamiana* leaves were agro-infiltrated with the wild-type NSm (NSm), Sw-5b, empty vector (EV) or NSm plus Sw-5b (NSm+Sw-5b). Only the area co-infiltrated with NSm and Sw-5b (Sw-5b+NSm) showed a cell-death response. The infiltrated leaf was photographed at 8 days post agro-infiltration (dpi). The expression of NSm and Sw-5b was driven by the 2x35S promoter in the binary vector pCambia1300. (b–e) Detection of H$_2$O$_2$ accumulation in the Sw-5b (b), NSm (c), Sw-5b+NSm (d), or EV (e) infiltrated leaves. After 3,3¢-diaminobenzidine (DAB) staining, the infiltrated leaves were examined under a light microscope at 200× magnification. (f) *N. benthamiana* leaves were agro-infiltrated with NSm$^*$-YFP (WT), NSm$^{A54-56}$-YFP (A54–56), NSm$^{A93-94}$-YFP (A93–94), NSm$^{A122-125}$-YFP (A122–125), NSm$^{A154}$-YFP (A154) or NSm$^{A269-274}$-YFP (A269–274) mutant in the presence of Sw-5b. The area labelled YFP was co-infiltrated with pCambia1300-YFP and Sw-5b, and used as a negative control. NSm$^*$ refers to the WT or its derivative mutants. The photograph was taken at 8 dpi. (g) Immunoblot detection of NSm-YFP and mutants accumulated in the infiltrated *N. benthamiana* leaves. Samples were taken from the *N. benthamiana* leaves at 48 h post infiltration. Extracts from the EV or pCambia1300-YFP (YFP)-infiltrated tissues were used as controls. Protein samples were separated in 10% SDS-PAGE by electrophoresis. The blot was detected using an anti-YFP polyclonal antibody (1 : 3000 dilution) followed by an AP-linked goat anti-rabbit antibody. The detection signal was visualized using NBT/BCIP substrate. Lanes 1–6 contain NSm$^{WT}$-YFP, NSm$^{A54-56}$-YFP, NSm$^{A93-94}$-YFP, NSm$^{A122-125}$-YFP, NSm$^{A154}$-YFP and NSm$^{A269-274}$-YFP samples. Lanes 7 and 8 contain EV and pCambia1300-YFP control samples, respectively. Ponseau S staining shows the loadings of each sample.
Fig. 2. Subcellular localization and intercellular movement of the wild-type (WT) and mutant NSms carrying alanine substitutions at amino acid residues 54–56 (A54–56), 93–94 (A93–94), 122–125 (A122–125), 154 (A154) or 269–274 (A269–274). (a–c) Co-expression of TSWV NSm-YFP with TMV MP-RFP. (d–r) Co-expression of individual mutants with TMV MP-RFP. TMV MP-RFP was used to show plasmodesmata in cell walls. NSm* refers to the WT or its derivative mutants. The YFP fluorescence signal is shown as green and the RFP fluorescence signal as red. Images were taken 48 h post co-infiltration (hpi), bar = 10 μm. (s–z) Intercellular movement of the wild-type (WT) and mutant NSms. To conduct cell-to-cell movement complementation assay, *N. benthamiana* leaves were agro-infiltrated with pCB301-CMV RNA3-MP::erGFP reporter alone (s) (diluted 1000-fold from OD600=0.5 of *Agrobacterium* tumefaciens cultures) or with undiluted *A. tumefaciens* cultures containing pCB301-CMV RNA1 and RNA2 (t), TSWV with the wild-type NSm (u), or TWSV with one of the mutant
infiltrated leaves (Fig. 1g). Therefore, we conclude that the function of NSm to target PD and to traffic between cells is uncoupled from its function to trigger Sw-5-mediated HR. Lopez et al. (2011) suggested that amino-acid substitution at position 118 (C118Y) or 120 (T120N) in the TSWV NSm might result in breaking of the Sw-5-mediated resistance in tomato. In 2014, Peiró et al. (2014) demonstrated that the presence of a tyrosine at position 118 or an asparagine at position 120 in NSm abolished the Sw-5-mediated HR. To further analyse these two amino acids, a single amino-acid substitution (C118Y or T120N) was introduced into the PD-nontargeting NSm mutant. We selected this mutant for further analysis because none of the five mutants described above were able to target PD. We also introduced the same amino acid substitutions into the wild-type NSm and used them as controls. These new NSm mutants (NSm\(^{C118Y}\) -YFP, NSm\(^{T120N}\) -YFP, NSm\(^{A54-56/C118Y}\) -YFP and NSm\(^{A54-56/T120N}\) -YFP) all had a YFP fused to the C-terminus. We then infiltrated individual new mutants together with TMV MP-RFP into N. benthamiana leaves. Co-infiltration of NSm\(^{C118Y}\) -YFP (Fig. 3a-c) or NSm\(^{T120N}\) -YFP (Fig. 3d-f) with TMV MP-RFP resulted in a co-localization of the two NSm mutants at PD. In contrast, co-infiltration of NSm\(^{A54-56/C118Y}\) -YFP (Fig. 3g-i) or NSm\(^{A54-56/T120N}\) -YFP (Fig. 3j-l) with TMV MP-RFP into N. benthamiana leaves did not yield co-localization of the two NSm fusions at PDs. In a separate study, the NSm mutants (NSm\(^{C118Y}\) , NSm\(^{T120N}\) , NSm\(^{A54-56/C118Y}\) or NSm\(^{A54-56/T120N}\) ) were introduced individually into the binary vector. These constructs all lacked the YFP gene and thus their ability to move between cells was determined using the CMV-based movement-complementation system described above. As shown in Fig. 3n-p, the CMV RNA3-MP::egfp reporter moved from cell to cell after co-expression with the NSm\(^{WT}\) , TSWV NSm\(^{C118Y}\) or NSm\(^{T120N}\). In contrast, co-expression of CMV RNA3-MP::egfp reporter with NSm\(^{A54-56}\), NSm\(^{A54-56/C118Y}\) or NSm\(^{A54-56/T120N}\) showed only single cells with green fluorescence (Fig. 3r-t). We then asked whether these NSm\(^{C118Y}\) , NSm\(^{T120N}\) , NSm\(^{A54-56/C118Y}\) or NSm\(^{A54-56/T120N}\) mutants were able to induce HR response. Individual mutants were co-infiltrated with the Sw-5b construct into N. benthamiana leaves. Results shown in Fig. 3u indicated that none of these mutants was able to elicit HR responses in the infiltrated N. benthamiana leaves. Immunoblot analysis confirmed that all four mutants were expressed to similar levels as the wild-type NSm in the infiltrated leaves (Fig. 3v). These results showed that the amino acids important for HR induction were not required for NSm PD targeting and cell-to-cell movement.

It is noteworthy to mention that the Avr domain for HR induction in many plant-virus-encoded proteins was uncoupled from the domains with other functions. For example, during TMV- and N-gene interaction, the helicase domain (p50) of the replicase is required for the induction of N-mediated defence response in tobacco, while the ATPase/helicase activity of this domain is not essential for HR induction (Erickson et al., 1999). Similarly, for the CMV and Cry interaction, the replicase domain in the CMV 2a was sufficient to induce HR in cowpea. While the conserved GDD motif in the 2a replicase is essential for viral replication, it is not required for the HR induction in cowpea (Hu et al., 2012). Moreover, the NSs-silencing suppressor of TSWV was shown to be critical for the induction of HR in Tsw-gene resistance pepper. However, the RNA-silencing-suppressor activity of this protein could be separated from its Avr activity (de Ronde et al., 2014). In this study, we showed that the cell-to-cell movement function of NSm could be separated from its function for HR induction. During the battle between virus invasion and plant defence, viruses utilize their encoded viral proteins for their replication, cell-to-cell and long-distance movement, and/or suppressing host basal defence (RNA silencing) to ensure their infection in the plant. The host plant has also evolved to build up its immune system to counter-attack virus invasion through recognizing viral proteins essential during the virus life cycle. Based on similar phenomena observed for many plant viruses, we propose that during the evolution of Resistance (R)–Avr protein recognition, the R protein produced by the plant immune system may itself have first evolved to recognize domains of viral-effector proteins rather than the domains involved in virus replication, movement or gene-silencing suppression. Compared to the strategy that inactivates viral functions needed in virus life cycles by the R protein, this R–Avr-recognition strategy should be immediate and highly efficient for the R protein to detect viruses. This may explain why the same phenomenon could be observed for many different viruses in the plant effector-triggered immunity (ETI) response. The recognition of plant R protein and viral-effector protein could also be mediated by indirect interaction(s). In this scenario, the viral avirulent protein may target or explore a host protein needed for a virus’s successful infection in a plant while this host protein may be under the surveillance of plant-resistance protein(s).

In summary, we demonstrated in this study that the PD targeting and cell-to-cell movement of TSWV NSm is uncoupled from its function for induction of HR response mediated by Sw-5b. Our conclusion is supported by two pieces of evidence. First, the NSm mutants used in this study were defective in PD targeting or intercellular trafficking but were capable of triggering HR. Second, introduction of a single amino-acid mutation, C118Y or T120N, identified previously from TSWV resistance-breaking isolates, into the movement-deficient NSm mutants, abolished their ability to induce HR. Soellick et al. (2000) and Paape et al.
Fig. 3. Subcellular localization, intercellular movement and hypersensitive response caused by the single amino-acid substitution mutants. Single amino acid substitutions (C118 and T120) were introduced individually into the wild-type NSm or the NSm<sup>A54–56</sup> mutant. <i>A. tumefaciens</i> GV3101 strain, containing NSm<sup>C118Y</sup>-YFP (a–c), NSm<sup>T120N</sup>-YFP (d–f), NSm<sup>A54–56 & C118Y</sup>-YFP (g–i), or NSm<sup>A54–56 & T120N</sup>-YFP (j–l), was co-infiltrated with the <i>A. tumefaciens</i>, harbouring the TMV MP-RFP into <i>N. benthamiana</i> leaves. NSm*-YFP indicates that the fluorescence signal (green) is from the WT NSm or various NSm derivatives. Fluorescence signal from TMV MP-RFP is shown as red. Images were taken at 48 h post co-infiltration (hpi), bar = 10 µm. The ability of each mutant to move cell to cell was determined through the complementation assay, using the CMV RNA3-MP::erGFP reporter as described above. <i>A. tumefaciens</i>, containing the pCB301-CMV RNA3-MP::erGFP reporter (diluted 1000-fold from OD<sub>600</sub>=0.5 of <i>A. tumefaciens</i> cultures), was agro-infiltrated alone (m) or co-infiltrated with <i>A. tumefaciens</i>,
containing pCB301-CMV RNA1 and RNA2 (OD_{560nm}=0.16), wild-type NSm (NSm^{WT}) (n), NSm^{C118Y} (o), NSm^{T120N} (p), p1300S empty vector (EV) (q), NSm^{A54–56} (r), NSm^{A54–66/C118Y} (s) or NSm^{A54–56/T120N} mutant (t) into N. benthamiana leaves. Green fluorescence in cells in the infiltrated leaves was examined at 48 hpi, size bars = 50 µm. The ability to cause cell death by the wild-type NSm (Wt) or individual single amino-acid substitution mutants was determined in the presence of Sw-5b (u). The A. tumefaciens GV3101 strain, harbouring the Sw-5b construct, was co-infiltrated with one of the A. tumefaciens cultures containing one of the NSm-substitution mutants [NSm^{C118Y} (C118Y), NSm^{T120N} (T120N), NSm^{A54–56} (A54–56), NSm^{A54–66/C118Y} (A54–66 C118Y) and NSm^{A54–56/T120N} (A54–56 T120N)] into leaves of N. benthamiana. Leaf areas co-infiltrated with the Sw-5b and EV were used as a controls. The infiltrated leaves were photographed at 8 days post agro-infiltration. Expression of the WT and mutant NSms in the infiltrated N. benthamiana leaves was confirmed via immunoblot analysis (o). Samples were taken from agro-infiltrated leaf tissues at 36 hpi. Proteins in these samples were separated in a 12.5 % SDS-polyacrylamide gel by electrophoresis. The NSm protein bands were detected with the anti-NSm mouse antibody followed by the IRD800-coupled goat anti-mouse IgG. Antigen–antibody interaction was then visualized with a Licor Odyssey scanner. Lanes 1–8 are NSm^{WT}, NSm^{C118Y}, NSm^{T120N}, EV, NSm^{A54–56}, NSm^{A54–66/C118Y}, NSm^{A54–56/T120N} and EV. Ponseau S staining gel shows the loading for each sample.

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