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The NSm proteins of phylogenetically related tospoviruses trigger Sw-5b–mediated resistance dissociated of their cell-to-cell movement function

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ABSTRACT

The cell-to-cell movement protein (NS_M) of tomato spotted wilt virus (TSWV) has been recently identified as the effector of the single dominant *Sw-5b* resistance gene from tomato (*Solanum lycopersicum* L.). Although most TSWV isolates shows a resistance-inducing (RI) phenotype, regular reports have appeared on the emergence of resistance-breaking (RB) isolates in tomato fields, and suggested a strong association with two point mutations (C118Y and T120N) in the NS_M protein. In this study the *Sw-5b* gene has been demonstrated to confer not only resistance against TSWV but to members of five additional, phylogenetically-related classified within the so-called "American" evolutionary clade, i.e., Alstroemeria necrotic streak virus (ANSV), chrysanthemum stem necrosis virus (CSNV), groundnut ringspot virus (GRSV), Impatiens necrotic spot virus (INSV) and tomato chlorotic spot virus (TCSV). Remarkably, bean necrotic mosaic virus (BeNMV), a recently discovered tospovirus classified in a distinct American subclade and circulating on the American continent, did not trigger a *Sw-5b*-mediated hypersensitive (HR) response. Introduction of point mutations C118Y and T120N into the NS_M protein of TSWV, TCSV and CSNV abrogated the ability to trigger *Sw-5b*-mediated HR in both transgenic-*N. benthamiana* and tomato isolines harboring the *Sw-5b* gene whereas it had no effect on BeNMV NS_M. Truncated versions of TSWV NS_M lacking motifs associated with tubule formation, cell-to-cell or systemic viral movement were made and tested for triggering of resistance. HR was still observed with truncated NS_M proteins lacking 50 amino acids (out of 301) from either the amino- or carboxy-terminal end. These data altogether indicate the importance of amino acid residues C118 and T120 in *Sw-5b*-mediated HR only for the NS_M proteins from one cluster of tospoviruses within the American clade, and that the ability to support viral cell-to-cell movement is not required for effector functionality.

1. Introduction

Tomato spotted wilt viruses (TSWV) rank high on the list of most devastating plant viruses worldwide, causing severe damage to many economically important agronomical crops. This virus was first reported in Australia by Brittlebank in 1919 (Stevens et al., 1991) but has re-emerged since the 1990's due to the worldwide expansion of its most important vector, the thrips *Frankliniella occidentalis* (Western flower thrips). Annual outbreaks are nowadays common in tropical and temperate climate zones, where new susceptible host species have been recorded (Pappu et al., 2009). *Tomato spotted wilt virus* belongs to the genus *Tospovirus*, which constitutes the only genus with plant-infecting members within the family *Bunyaviridae* (King et al., 2012). Based on their genome, tospoviruses are grouped in four main phylogenetic lineages, two with an "American" origin and two with a Eurasian origin (de Oliveira et al., 2012). With the description of more tospovirus species, other clades have been suggested (Lima et al., 2016). Tospoviruses have a tripartite RNA genome of negative and ambisense polarity that contains five open reading frames (ORFs) coding for 4 structural (L, Gn, Gc, N) and 2 non-structural (NS_M and NS_s) proteins. Current efforts for tospovirus disease management involves the implementation of cultural, phytosanitary, and chemical approaches (Pappu et al., 2009). To reduce the costs and environmental damage associated with the use of insecticides, a great focus has been put on

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obtaining genetically resistant cultivars as an essential and active component of disease management strategies.

The *Sw-5* gene cluster from *Solanum peruvianum* is the only known resistance source available for commercial breeding of tomato against TSWV (Boiteux and Giordano, 1993; Stevens et al., 1991). Although five *Sw-5* paralogs, named *Sw-5a* to *Sw-5e,* have been cloned from this gene cluster from resistant tomato, only *Sw-5b* has been shown to confer resistance against TSWV (Spassova et al., 2001; De Oliveira et al., 2016). The *Sw-5b* gene codes for a member of the NB-LRR receptor protein family with a solanaceae domain (SD)-coiled-coil (CC) N-terminus, nowadays also known as the SD-CNL class (De Oliveira et al., 2016; Chen et al., 2016). Recently, the NS_M of TSWV has been identified as the avirulence determinant (Avr)/effector of *Sw-5b*, inducing a hypersensitive cell death response (HR) in both *Sw-5b*-transformed *N. benthamiana* and in *Sw-5*-resistant tomato isolines (Hallwass et al., 2014; Peiro et al., 2014). The NS_M protein is a non-structural protein that facilitates the spread of tospoviruses in plants by cell-to-cell movement of non-enveloped nucleocapsids via plasmodemata (Kormelink et al., 1994; Storms et al., 1995). To this end, the protein forms tubules protruding from plasmodesmata from the infected- into the neighboring healthy cells and modifies the plasmodesmata structure. The TSWV NS_M protein consists of 301 amino acids and domains required for tubule formation, cell-to-cell movement and long distance movement have been mapped in earlier studies (Leastro et al., 2017; Li et al., 2009; Silva et al., 2001).

During the last two decades, resistance-breaking (RB) TSWV isolates have emerged, most likely due to a high selection pressure on the virus, thereby threatening the durability of *Sw-5b*-resistance (Gordillo et al., 2008). While this has provoked increased efforts to screen germplasms and breeding lines for resistance, attempts to further unravel the underlying molecular mechanisms linked to resistance breakdown has thus far received limited attention. Based on multiple sequence alignments of 18 different TSWV isolates, (Lopez et al., 2011) suggested that resistance conferred by *Sw-5b* can be overcome by two independent single-amino acid substitutions (C118Y and T120N) in the NS_M protein as a result of positive selection. In a recent study this was confirmed experimentally (Peiro et al., 2014). Given the diversity of characterized tospoviruses that trigger *Sw-5b*, resistance inducing (RI) isolates, and the regular emergence of RB strains in tomato fields, a considerable gap in knowledge still exists on which topological region of NS_M is required for the interaction and triggering of resistance.

In this study we have analyzed the broadness of resistance spectrum from *Sw-5b* against tospoviruses from the American (sub) clades and tested NS_M mutants/truncations on triggering of resistance to identify and/or confirm the involvement of certain amino acid residues and domains for effector functionality. The results indicate the broad functionality of the 118–120 residues that confer RI and RB to distinct tospoviruses and that avirulence maps to the central core region of NS_M, most conserved among tospoviruses.

2. Materials and methods

2.1. Virus and plant maintenance

The inoculums containing the tospoviruses ANSV, BeNMV, CSNV, GRSV, INSV, TCSV and TSWV isolates BR01, Gn1nL2 and GRAU, used in this work were provided by research groups in Brazil, Spain and Netherlands. All tospoviruses were maintained in susceptible *Nicotiana benthamiana* plants or *Physalis pubecens* (for BeNMV) by mechanical inoculation using phosphate buffer (0,01 M monobasic sodium phosphate and 1% sodium sulphate, pH 7,0).

N. benthamiana plants (wild type and transformed with *Sw-5b* gene), *S. lycopersicum* cultivar "Santa Clara" (highly susceptible to tospoviruses) and *S. lycopersicum* isogenic line "CNPH LAM 147" (tospovirus resistant near-isogenic line harboring the *Sw-5b* gene) were maintained in FITOTRON® plant growth chambers under conditions of 23 °C day–18 °C night, 70% humidity with 16 h light/8 h dark regime.

The transgenic *N. benthamiana* plant transformed with the *Sw-5b* gene (from here after referred as Nb/*Sw-5b*) was generated from infiltration of *Agrobacterium tumefaciens* strain LBA44A4 containing the *Sw-5b* gene copy. The tospovirus resistant near-isogenic line "CNPH LAM 147" (denoted LAM147) was generated by crossing of the *S. lycopersicum* cultivar "Santa Clara" with the resistant cultivar "Viradoro". All handling for obtaining the Nb/*Sw-5b* and LAM147 plants was previously described by Hallwass et al. (2014).

2.2. DNA manipulation

For mutational assays, the NS_M gene of the viruses BeNMV, CSNV and TCSV was obtained by reverse transcription polymerase chain reaction (RT-PCR) from RNA extracted of leaves inoculated with the distinct tospoviruses (Sambrook et al., 1989). All NS_M genes were cloned into the vector pGEMT-Easy using primers with specific restriction sites *PciI*/*Nhe*I, except for the CSNV NS_M, for which the *BspHI*/*Nhe*I restriction sites were used. The NS_M gene of the tospovirus isolates $TSWV_{Grau}$ and $TSWV_{Gr1nL2}$ was provided by Ana Peiró (Intituto de Biología Celular y Molecular de Plantas – IBMCP/Spain).

To fuse the hemagglutinin (HA) sequence at the C-terminus of NS_M proteins, the amplified genes were subcloned in the pSK-35S-MPTMV:HA construct (Peiro et al., 2014), replacing the tobacco mosaic virus (TMV) MP gene. The resultant clones pSK-35S-NS_M:HA-PoPit (Leastro et al., 2015) contained the corresponding NS_M fused to the HA epitope under the control of 35S promoter from cauliflower mosaic virus (CaMV) and the terminator from the potato proteinase inhibitor II, PoPit.

The nucleotide mutations were performed by inverted PCR with the enzyme Phusion highly-fidelity DNA polymerase (Life Technologies™). As template for PCR, the vectors pSK-35S-NS_M:HA-PoPit (Leastro et al., 2015) were used and re-amplified with specific primers containing nucleotide substitutions correspondent to the 118–120 residues. A serial dilution of plasmids containing the NS_M genes was performed to decrease the possibility of recloning of non-mutated plasmid used as template for inverted-PCR. Then, phenolization (Sambrook et al., 1989) of PCR-vector product was performed and finally the phosphorylation step (Kinase enzyme BioLabs®) and ligation (Ligase enzyme BioLabs®) of the vectors, following the manufacturer's recommendations. All DNA manipulations were confirmed by plasmid DNA sequencing.

All resulting 35S-NS_M:HA expression cassettes non-mutated constructs: 35S-NS_MBeNMV$_{wt}$:HA, 35S-NS_MCSNV$_{wt}$:HA, 35S-NS_MTCSV$_{wt}$:HA, 35S-NS_MTSWV$_{Gr1nL2wt}$:HA and mutated: 35S-NS_MBeNMV$_{mut}$:HA, 35S-NS_MCSNV$_{mut}$:HA, 35S-NS_MTCSV$_{mut}$:HA and 35S-NS_MTSWV$_{mut}$:HA were subcloned into the pMOG800 binary vector by using the restriction enzyme *Xho*I.

For construction of deletions, the pDONR207 harboring the NS_M gene from the TSWV isolate BR-01 (Hallwass et al., 2014) was used as template for amplification of truncated gene versions by PCR. Primers annealing at the beginning (for C-terminal deletion constructs) or at the end (for N-terminal deletion constructs) of the NS_M gene were combined with the following primers to truncate the coding sequence: N-25aa-F GGGGACAAGT TTGTACAAA AAAGCAGGCT TCGAAGGAGA TAGAACCATG CATAATGGCA GTGTTGAAGTC; N-50aa-F GGGGACAAGT TTGTACAAAA AAGCAGGCTT CGAAGGAGAT AGAACCATGG CATCCAAAGG AAAGATACTG; N-100aa-F GGGGACAAGT TTGTACAAAA AAGCAGGCTT CGAAGGAGAT AGAACCATGC TTTTTGTTGG CAACGGAAAG; C-25aa-R GGGGACCACT TTGTACAAGA AAGCTGGGTC CTATGAGCTC AGTTCTTTAA GCTG; C-50aa-R GGGGACCACT TTGTACAAGA AAGCTGGGTC CTAAATGAAT GCCTGAGATC TAGC; C-100aa-R GGGGACCACT TTGTACAAGA AAGCTGGGTC CTAACACATC AAATGCAGCT GACA. The PCR products were then recombined with pDONR207 by BP clonase (Invitrogen™). Finally, the resulting entry vectors were recombined with pK2GW7 (Karimi et al., 2002) by LR clonase (Invitrogen™) and used to transform the *Agrobacterium*