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The functional analysis of distinct tospovirus movement proteins (NS_M) reveals different capabilities in tubule formation, cell-to-cell and systemic virus movement among the tospovirus species

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ABSTRACT

The lack of infectious tospovirus clones to address reverse genetic experiments has compromised the functional analysis of viral proteins. In the present study we have performed a functional analysis of the movement proteins (NS_M) of four tospovirus species *Bean necrotic mosaic virus* (BeNMV), *Chrysanthemum stem necrosis virus* (CSNV), *Tomato chlorotic spot virus* (TCSV) and *Tomato spotted wilt virus* (TSWV), which differ biologically and molecularly, by using the *Alfalfa mosaic virus* (AMV) model system. All NS_M proteins were competent to: i) support the cell-to-cell and systemic transport of AMV, ii) generate tubular structures on infected protoplast and iii) transport only virus particles. However, the NS_M of BeNMV (one of the most phylogenetically distant species) was very inefficient to support the systemic transport. Deletion assays revealed that the C-terminal region of the BeNMV NS_M, but not that of the CSNV, TCSV and TSWV NS_M proteins, was dispensable for cell-to-cell transport, and that all the non-functional C-terminal NS_M mutants were unable to generate tubular structures. Bimolecular fluorescence complementation analysis revealed that the C-terminus of the BeNMV NS_M was not required for the interaction with the cognate nucleocapsid protein, showing a different protein organization when compared with other movement proteins of the '30K family'. Overall, our results revealed clearly differences in functional aspects among movement proteins from divergent tospovirus species that have a distinct biological behavior.

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1. Introduction

After the establishment of the infection in the initial infected cells, plant viruses invade the neighbor cells via the cell wall connections known as plasmodesmata (PD), the so-called cell-to-cell transport (Fernandez-Calvino et al., 2011; Lucas, 2006), and reach distal parts of the plant through the vascular tissue, a process denominated systemic transport (Carrington et al., 1996; Lazarowitz, 1999; Lazarowitz and Beachy, 1999; Pallas and Garcia, 2011; Ueki, 2007; Waigmann et al., 2004). For this purpose, the viruses express one or a few movement protein(s) (MPs). The MPs can be divided into two main categories based on the degree of structural changes that they induce in the PD (Benitez-Alfonso et al., 2010; Niehl and Heinlein, 2011; Scholthof, 2005). The first one is that represented by the MP of the *Tobacco mosaic virus* (TMV) that interacts with the viral RNA and facilitates the transport of a ribonucleotide complex through the PD without causing any visual changes (Heinlein and Epel, 2004; Niehl and Heinlein, 2011; Wolf et al., 1989). The other category is represented by the MP of *Cowpea mosaic virus* (CPMV) that forms tubular structures that drastically modify the PD and facilitate the virus passage in the form of virions (Ritzenthaler and Hofmann, 2007). In spite of the clear differences observed among the two transport mechanisms, both MPs have been assigned to the '30K superfamily' (Melcher, 2000; Mushegian and Elena, 2015).

Tospovirus is the only genus of the family *Bunyaviridae* that includes plant-infecting viral species. Their genome consists of three single-stranded RNA segments denoted S RNA (small), M RNA (medium) and L RNA (large), respectively, having the first two an ambisense coding strategy and the L segment a negative polarity. The L segment encodes an RNA-dependent RNA polymerase (de Haan et al., 1991). Both M and S RNA have two "open reading frames" (ORFs) separated by an intergenic region (IRG). The ORFs of the S segment encode the non-structural NS_S protein, identified as an RNA silencing suppressor (Takeda et al., 2002) and the N protein involved in the formation of viral ribonucleocapsids (NPs). In

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the M segment, the ORFs encode the precursor of the G_n and G_c glycoproteins that are localized in the viral particle envelope and that are important for tospovirus transmission mediated by thrips (Ribeiro et al., 2009; Sin et al., 2005), and another non-structural protein (NS_M), involved in the viral movement (Kormelink et al., 1994) and, more recently, proved to work as an avirulence determinant associated with the Sw-5 resistance gene (Hallwass et al., 2014; Peiro et al., 2014a). In some aspects, the tospovirus are still the less understood genus of the *Bunyaviridae* family mainly due to the lack of infectious clones to address reverse genetic analysis. Part of such limitations have been overcome by using the *Alfalfa mosaic virus* (AMV) model system, which permits the functional exchangeability of MPs assigned to the '30K family' (Fajardo et al., 2013; Melcher, 2000; Peiro et al., 2014b; Sanchez-Navarro et al., 2006).

The tospovirus cell-to-cell transport occurs through tubular structures derived from the NS_M (Pappu et al., 2009). The viral complexes transported through such structures have been proposed to be nonenveloped ribonucleocapsids, via a direct interaction between NS_M-N proteins (Kormelink et al., 1994; Storms et al., 1995). However, the observation that the NS_M of TSWV supports the local and systemic transport of a TMV CP deletion mutant (Lewandowski and Adkins, 2005), suggest that NS_M could transport other complexes different than ribonucleocapsids. Although, it is expected that tospovirus transport mechanism would be conserved among the 28 species of the genus (Adkins, 2000; Pappu et al., 2009), comparisons of virus species within the genus, showed significant amino acids differences of the NS_M proteins that could be related to different biological features such as virus movement. Tospoviruses display a considerable degree of biological diversity although phylogeny studies based on nucleocapsid protein amino acid sequences grouped members of this genus into two main clades, such as those from Asia or the Americas (Adkins, 2000; Bezerra et al., 1999; de Avila et al., 1993; Lebas and Ochoa-Corona, 2007; Pappu et al., 2009; Persley et al., 2006). Recently, a new evolutionary lineage within the genus *Tospovirus* has been observed after the characterization of *Bean necrotic mosaic virus* (BeNMV) and *Soybean vien necrosis-associated virus* (SVNaV) (de Oliveira et al., 2012; Zhou et al., 2011). Viral movement proteins facilitate the translocation of plant viruses among cells and through the plant and may play a crucial role in host specificity and in modulating plant physiology (Garcia and Pallas, 2015). Based on these aspects, the aim of this study was to characterize the functional features of the NS_M of a very distant phylogenetically related tospovirus (BeNMV) infecting mainly leguminous plants with three tospovirus members grouped in the same Americas clade and that affect many solanaceae species (*Chrysanthemum stem necrosis virus* – CSNV, *Tomato chlorotic spot virus* – TCSV and *Tomato spotted wilt virus* – TSWV). Thus, we seek to compare contrasting tospoviruses that naturally show different capabilities to infect systemically and to move in distinct plant hosts. In addition, elucidating viral movement mechanisms will open new scope for understanding the molecular processes of the viral infection, an aspect that will contribute in the development of anti-viral treatments.

Our results showed that all NS_M proteins were functional in the AMV system either for the local and systemic transport. We observed common properties between all analyzed NS_M proteins but also clear differences between the NS_M of a virus biologically distinct (BeNMV), in comparison to the others (CSNV, TCSV and TSWV) biologically more similar. Overall, our results clearly demonstrate differences in aspects of the functionality among the NS_M proteins in a viral genus. These differences could reveal a distinct tospovirus evolution and/or adaption, which could have involvement with to their distinct efficiency to promote the cell-to-cell and systemic movement among the host species.

2. Materials and methods

2.1. DNA manipulation

2.1.1. Constructs for analyses of cell-to-cell and systemic transport

For the analysis of the cell-to-cell transport, a modified infectious AMV cDNA 3 clone, which expresses the green fluorescent protein (GFP) (pGFP/A255/CP) (Sanchez-Navarro et al., 2001), was used to exchange the N-terminal 255 amino acids of the AMV MP gene with the corresponding NS_M gene of *Bean necrotic mosaic virus* (BeNMV; GenBank: YP_006468901.1), *Chrysanthemum stem necrosis virus* (CSNV; GenBank: AAK84656.1), *Tomato chlorotic spot virus* (TCSV; GenBank: AAK84655.1) and *Tomato spotted wilt virus* (TSWV; GenBank: HM015513). The NS_M genes were amplified from the pGEMT-Easy vectors (de Oliveira et al., 2012; Silva et al., 2001), with specific primers containing the *Nco*I, *Pci*I, *BspH*I and *Nhe*I restriction sites (Fig. 1), to generate the constructs pGFP/BeNMV:A44/CP, pGFP/CSNV:A44/CP, pGFP/TCSV:A44/CP and pGFP/TSWV:A44/CP (referred as pGFP/NRB:A44/CP in Peiro et al., 2014a), respectively. The resultant NS_M proteins are fused to the C-terminal 44 amino acids (A44) of the AMV MP. Additionally, the NS_M genes were introduced in a chimeric infectious cDNA 3 clone of AMV, lacking the GFP gene and containing the NS_M gene of TSWV (pNRB:A44/CP; Peiro et al., 2014a) by exchanging the *Nco*I-*Nhe*I fragment. The resultant chimeric plasmids were referred as pBeNMV:A44/CP, pCSNV:A44/CP, pTCSV:A44/CP and pTSWV:A44/CP. DNA amplifications was performed with the Platinum Taq DNA polymerase following the manufacturer's specifications (Invitrogen^{TM}).

The pGFP/NS_M:A44/CP constructs were modified to introduce a CP gene lacking the C-terminal 14 (mutant CP-N206) and defective in virus formation (Tenllado and Bol, 2000). The introduction of the corresponding mutated CP genes was performed by exchanging the *Nhe*I-*Pst*I fragment obtained from mutants pGFP/BMV:A44/CP-N206 (Sanchez-Navarro et al., 2006), to generate the constructs pGFP/BeNMV:A44/CP-N206, pGFP/CSNV:A44/CP-N206, pGFP/TCSV:A44/CP-N206 and pGFP/TSWV:A44/CP-N206.

For the functional analysis of the C-terminal NS_M mutant proteins, the corresponding truncated NS_M genes were amplified using specific primers containing the restriction sites *Pci*I, *BspH*I, *Nco*I and *Nhe*I, as specified in Fig. 1. The amplified NS_M genes were digested with the corresponding enzymes and introduced in the pGFP/A255/CP (Sanchez-Navarro et al., 2001) or pNRB:A44/CP (Peiro et al., 2014a), previously digested with the *Nco*I-*Nhe*I restriction enzymes.

2.1.2. Constructs for tubule formation

Tubule formation in protoplasts was analyzed with a chimeric AMV RNA3 in which the MP contains the GFP fused at its C-terminus (construct pMP:GFP/CP; Sanchez-Navarro et al., 2001). Full length or mutated NS_M genes were amplified with specific primers containing the restriction sites *Pci*I, *BspH*I, *Nco*I and *Nhe*I (Fig. 1). The resultant PCR products were digested with the corresponding enzymes and introduced in the pMP:GFP/CP construct by exchanging the AMV MP using the *Nco*I-*Nhe*I restriction sites, to generate the constructs: pBeNMV:GFP/CP, $pBeNMV_{295}$:GFP/CP, $pBeNMV_{300}$:GFP/CP, pCSNV:GFP/CP, $pCSNV_{298}$:GFP/CP, pTCSV:GFP/CP, $pTCSV_{298}$:GFP/CP, pTSWV:GFP/CP and $pTSWV_{298}$:GFP/CP.

2.1.3. Constructs for *in vivo* protein-protein interaction (BiFC)

To evaluate the *in vivo* interaction between the BeNMV NS_M mutants with the cognate N protein, the mutated NS_M genes corresponding to the N-terminal 295 or 300 amino acids (aa)

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