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The movement proteins (NSm) of distinct tospoviruses peripherally associate with cellular membranes and interact with homologous and heterologous NSm and nucleocapsid proteins

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ABSTRACT

Tospovirus is the only genus containing virus species which infect plants in the *Bunyaviridae* family. The aims of this study were to understand the *in vivo* membrane association of the movement protein (NSm) of the tospovirus species *Bean necrotic mosaic virus*, *Chrysanthemum stem necrosis virus*, *Tomato chlorotic spot virus* and *Tomato spotted wilt virus* and the homologous and heterologous interactions among NSm and nucleocapsid protein (N). The results obtained by bimolecular fluorescence complementation (BiFC) assay and chemical treatments after membrane fractionation revealed that the four NSm proteins are associated with the biological membranes with the N- and C-termini oriented to the cytoplasm. BiFC analysis for protein–protein interactions showed: i) dimer formation for all NSm and N proteins; ii) interaction between NSm and the cognate N and iii) heterologous interactions between the NSm and N proteins. The implications of these interactions in the life cycle of tospoviruses are discussed.

Introduction

Tospovirus is the only genus of the family *Bunyaviridae* that includes plant-infecting viral species. The type species, *Tomato spotted wilt virus* (TSWV), is currently ranked among the top 10 most economically important plant viruses worldwide with significant losses in agronomic production (Adkins, 2000; Scholthof et al., 2011). 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. Tospovirus genome presents five "open reading frames" (ORFs) that encode four structural and two nonstructural proteins (Pappu et al., 2009). The L segment has a complete negative polarity and contains an ORF encoding an RNA-dependent RNA polymerase (de Haan et al., 1991). Both M and S RNA have two ORFs separated by an intergenic region (IRG). The ORF present in the viral sense of the S RNA segment encodes the non-structural NSs protein, identified as an RNA silencing suppressor (Takeda et al., 2002) and the ORF located in the complementary viral RNA encodes the N protein involved in the formation of viral ribonucleocapsids (NPs). The ORF located in the complementary viral M RNA encodes the precursor of the Gn and Gc glycoproteins that are localized in the viral particle envelope and that are important for tospovirus transmission mediated by thrips in a circulative and propagative mode (Ribeiro et al., 2009; Sin et al., 2005). The ORF in the viral RNA sense encodes a non-structural protein (NSm) involved in the short distance (cell-to-cell) and systemic movements (Pappu et al., 2009) and, more recently, proved to work as an avirulence determinant (Hallwass et al., 2014; Peiro et al., 2014a).

Cell-to-cell and systemic transports of viruses are provided *via* viral movement proteins (MPs) that generally associate themselves to host factors and other viral components (Aparicio et al., 2010; Dickert et al., 2004; Pouwels et al., 2004; Sanchez-Navarro et al., 2006; Wolf et al., 1989). Genetic and molecular biology studies have demonstrated that those proteins, denoted movement proteins (MPs), interact with the plasmodesma (PD) enhancing its exclusion limit and allow the virus passage (Wolf et al., 1989). 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 the ribonucleotide complex through the PD without causing any visual changes (Citovsky et al., 1992; Heinlein and Epel, 2004; Kawakami et al., 2004; McLean et al., 1995; Wolf et al., 1989). The other category

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is represented by the MP of *Cowpea mosaic virus* (CPMV) that forms tubular structures that drastically modify the PD and facilitates the virus passage in the form of virions (Kasteel et al., 1993; van Lent et al., 1990; Wellink and Vankammen, 1989). MPs are commonly found in association with different cell components including cytoskeleton elements, endoplasmic reticulum (ER) or cellular membranes (Heinlein and Epel, 2004; Kormelink et al., 1994; Lucas, 2006; Nelson and Citovsky, 2005; Pallas and Garcia, 2011; Wolf et al., 1989). The MP of TMV, the type member of the 30K family (Melcher, 2000), has been recently proposed to be associated to the cytosolic surface of the ER, without spanning the membrane (Peiro et al., 2014b). It has been proposed that this topology could be the general rule to other members of the 30K family, including the MP of tospovirus, although no data have been reported demonstrating this. In addition to the association with biological membranes, the MPs have been described to require in some instances the cognate nucleocapsid proteins, to allow the transport of specific coat protein (CP) complexes during virus movement (Aparicio et al., 2010; Nagano et al., 1997; Sanchez-Navarro and Bol, 2001; Sanchez-Navarro et al., 2006; Takeda et al., 2004). Viral MPs are not only key players in the virus movement but can have a significant contribution in the symptom development (Garcia and Pallas, 2015) or host susceptibility (Amari et al., 2012).

Studies on the subcellular localization of tospovirus proteins demonstrated the existence of different sites and forms of aggregation (German et al., 1992; Schmaljohn and Nichol, 2001; Lacorte et al., 2007; Kormelink et al., 2011; Dietzgen et al., 2012). For N protein is observed the formation of dispersed aggregates all over the cell. Similar cytoplasmic aggregates have been previously detected when N and C terminal ends of the GFP were fused to the N protein of TSWV in *Nicotiana benthamiana* leaves (Lacorte et al., 2007). The same type of aggregates were observed in BiFC assay with the N of INSV. Copies of N protein generate multimers in association with viral RNA to form RNP (German et al., 1992; Schmaljohn and Nichol, 2001). NSm expression in plant was studied for two different tospoviruses: the NSm of TSWV associates with ribonucleoprotein complexes (RNPs) located in the tubular structure of PD (Kormelink et al., 2011) meanwhile the NSm of INSV forms aggregates at the cell surface (Dietzgen et al., 2012). In addition, the N and NSm of INSV interact *in vivo*, showing peripherial localization and the formation of aggregates of different sizes, dispersed in the cytoplasm (Dietzgen et al., 2012). The interaction between N and NSm proteins offers a molecular basis for understanding the factors involved in the assembly of viral particles along with its movement (Kormelink et al., 2011; Pappu et al., 2006; Soellick et al., 2000). These observations allow us to predict that a particular viral protein could facilitate the infection caused by another virus that in a natural infection would be partially or totally restricted (Bag et al., 2012).

Interactions among viruses seem to be a common feature in nature. Mixed infections under natural conditions between viral species of the same genus may provide greater genetic diversity, favoring virus infection, genetic reassortment and recombination (Brown et al., 2002; Idris and Brown, 2004; Idris et al., 2008). Mixed infections and genetic reassortments have been also reported for the genus *Tospovirus* (García-Cano et al., 2006; Webster et al., 2011); however, these reports are scarce. A true synergistic interaction was observed between TSWV and the crinivirus *Tomato chlorosis virus* (ToCV), in which the ToCV infection caused the overcoming of resistance in tomato plants by the TSWV (García-Cano et al., 2006). There are also reports of mixed infections between the two tospovirus species: *Iris Yellow Spot Virus* (IYSV) and TSWV are present in 7% of field tested onion plants (Mullis et al., 2004). In this case, the nature and extension of co-infection were tested, indicating that TSWV may facilitate the systemic transport of IYSV in *Datura stramonium*, a host that restricts the movement of IYSV to inoculated leaves (Bag et al., 2012). A more extensive study of interactions between viral proteins of distinct species or with host proteins may shed light on the nature of multiple and mixed infections and generate a better understanding of potential synergism and/or antagonism processes resulting from tospovirus mixed infections. In this sense, we selected the tospovirus MPs as critical components during virus transport in which compatible interactions with either others MP and/or N proteins could represent a synergistic effect in mixed infections. Also, we selected four biologically different tospovirus species with a narrow (*Bean necrotic mosaic virus*, BeNMV) or a large (TSWV; *Chrysanthemum stem necrosis virus*, CSNV; *Tomato chlorotic spot virus*, TCSV) range of host plants.

In this context, the present study aimed to analyze first, the MP (here after NSm) membrane topology of the four tospovirus species BeNMV, CSNV, TCSV and TSWV and secondly, the capacity of the different NSm to interact with homologous and heterologous tospovirus proteins (NSm and N). Cell fractionation and biochemical treatments assays of the NSm expressed *in planta*, together with the results of bimolecular fluorescence complementation (BiFC) studies, permit to propose that the four NSm are peripherally associated to the cytosolic face of the ER membranes in living plant cells. Finally, the NSm interactions analysis generated information that can facilitate a better interpretation of viral proteins interactions, strengthening the understanding of mixed infection processes.

Results

NSm protein of tospovirus peripherally associates with membranes in vivo.

In silico analysis of NSm proteins from distinct tospovirus belonging to the American Clade predicted the presence of a hydrophobic region preceded by an aspartate residue (D-motif), in the central region of the protein (Silva et al., 2001). Based on this prediction, and in order to analyze the membrane association of tospovirus NSm in the experimental host *N. benthamiana*, we proceeded to transiently express the NSm fused to the HA epitope, by infiltrating *Agrobacterium tumefaciens* cultures carrying the corresponding pMOG800 35S-NSm:HA plasmids. At three days post-infiltrations, total proteins extracts were centrifuged at 100,000*g* to generate a membrane-rich fraction that was subjected to the above-described chemical treatments. To identify the type of interaction, we first washed the membrane-rich fraction with sodium carbonate (pH 11.5), a treatment that is known to render microsomes into membranous sheets, releasing soluble luminal proteins (Peremyslov et al., 2004). The results showed that the tospovirus NSm remained associated with the membranous fraction after sodium carbonate treatment (94% for BeNMV NSm and 100% for CSNV, TCSV and TSWV NSm in the pellet fraction; Fig. 1). Next, we washed the membranes with 4 or 7 M urea, a treatment that should release all polypeptides from the membrane, except the integral membrane proteins (Martínez-Gil et al., 2009). Both treatments (4 M and 7 M urea) led to the detection of NSm in the soluble fraction (9–18% and 54–92%, respectively), in contrast to the control experiments performed with the HA-tagged Lep as an integral membrane protein fashion that showed Lep accumulation exclusively in the membranous fractions following these treatments (Fig. 1). These results indicate that the four tospovirus NSm behave as membrane associated proteins rather than being fully integrated membrane proteins.

Membrane association of tospovirus NSm in living plant cells

The above results indicated that the four tospovirus NSm are associated, but not integrated, with the biological membranes. However, these results could not discern in which subcellular compartment the NSm are exposed. To answer this question, we performed a BiFC