Document downloaded from:

http://hdl.handle.net/10251/151320

This paper must be cited as:

Leastro, MO.; Pallás Benet, V.; Resende, RO.; Sanchez Navarro, JA. (2017). The functional analysis of distinct tospovirus movement proteins (NSM) reveals different capabilities in tubule formation, cell-to-cell and systemic virus movement among the tospovirus species. Virus Research. 227:57-68. https://doi.org/10.1016/j.virusres.2016.09.023

The final publication is available at

https://doi.org/10.1016/j.virusres.2016.09.023

Copyright Elsevier

Additional Information

Manuscript Details

Manuscript number	VIRUS_2016_381
Title	The Functional Analysis of Distinct Tospovirus Movement Proteins (NSM) Reveals Different Capabilities in Tubule Formation, Cell-to-Cell and Systemic Virus Movement Among the Tospovirus species.
Article type	Research Paper

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 (NSM) 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 NSM 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 NSM 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 NSM, but not that of the CSNV, TCSV and TSWV NSM proteins, was dispensable for cell-to-cell transport, and that all the non-functional C-terminal NSM mutants were unable to generate tubular structures. Bimolecular fluorescence complementation analysis revealed that the C-terminus of the BeNMV NSM 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.

Keywords	Cell-to-Cell and Systemic Movement, NSM Protein, NSM functionality, Tospovirus, Tubule formation, AMV system.

Corresponding Author	Jesus Sanchez-Navarro
Corresponding Author's Institution	Instituto de Biologi-a Molecular y Celular de Plantas
Order of Authors	Mikhail O. Leastro, Vicente Pallás, Renato O. Resende, Jesus Sanchez-Navarro
Suggested reviewers	Richard Komerlink

Submission Files Included in this PDF

File Name [File Type]

COVER LETTER VR.docx [Cover Letter]

Highlights.docx [Highlights]

Leastro et al Final Version VR.docx [Manuscript File]

Figure 1.tif [Figure]

Figure 2-01.tif [Figure]

Figure 3-01.tif [Figure]

Figure 4-01.tif [Figure]

Figure 5-01.tif [Figure]

Figure 6-01.tif [Figure]

Figure 7-01.tif [Figure]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

Valencia July 4, 2016

Editor of Virus Research,

Dear Editor,

We are submitting this manuscript entitled **“The functional analysis of distinct tospovirus movement proteins (NS_M) reveals different capacities in tubule formation, cell-to-cell and systemic virus movement among the tospovirus species.”** by Leastro *et al.* to *Virus Research*.

Our research group has been working with the AMV expressing system and virus movement proteins for several years and now, together with the Brazilian group working with tospovirus, we jointed efforts trying to elucidate tospovirus movement and protein interaction during virus infection. *Tospovirus* is the only plant-infecting genus in the *Bunyaviridae* family and despite of all the work carried out worldwide, *Tospovirus* is still the less understood genus of the family. Remarkably, lack of a reverse genetics system, which is available for other plant viruses, has hampered tospovirus research, as well as our understanding of protein functions and protein interactions during virus infection.

Therefore, joining our experiences, here we performed functional analysis of four tospovirus movement proteins (NS_M) that differs in the host range. We observed common properties between all analyzed NS_M proteins but also clear differences between the NS_M of virus with a narrow or a broad spectrum of host range. These differences could reveal a distinct tospovirus evolution and/or adaption concerning to their ability to move among distinct hosts, but also will open interesting research lines for further analysis. Likewise, we observed that none of the four NS_M proteins was able to transport non encapsidated viral nucleic acids, a result that differs completely to previous data showing that the *Tomato spotted wilt virus* NS_M protein, supports the local and systemic transport of a *Tobacco mosaic virus* coat protein deletion mutant. This aspect is amply commented in the Discussion section. Finally, we observed that the C-terminus of the four NS_M proteins behaves differently when compared to other movement proteins of the 30K family, since this portion of the protein is required for the functionality of the protein and is not required for an interaction with the nucleocapsid (N) protein.

We believe that this manuscript will be of interest not only to virologists, but also to those readers interested in the virus-host interaction and the putative role of the movement proteins. We hope all these traits convey this work suitable for publication in *Virus Research.*

Sincerely,

Jesús Angel Sanchez-Navarro
Corresponding author
Instituto de Biología Molecular y Celular de Plantas
Universidad Politécnica de Valencia-CSIC
Valencia, Spain

1. Four tospovirus movement protein allowed the transport of Alfalfa mosaic virus
2. The four movement proteins transported only virus particles in the AMV system
3. The C-terminus of the four movement proteins behaves differently
4. The C-terminus is not required for the interaction with the nucleocapsid protein

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.

Mikhail O. Leastro[a], **Vicente Pallás**[b], **Renato O. Resende**[a] **and Jesús A. Sánchez-Navarro**[b*]

[a] Departamento de Biologia Celular, Universidade de Brasília, 70910-900 Brasília, Brasil.
[b] Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de investigaciones Científicas-Universidad Politécnica de Valencia, Valencia, Spain.

Leastro, M.O.: m.leastro@gmail.com
Pallás, V.: vpallas@ibmcp.upv.es
Renato, R.O.: rresende@unb.br
Sánchez-Navarro, J.A*.: jesanche@ibmcp.upv.es.

Author for correspondence: Jesús Ángel Sánchez-Navarro
E-mail: jesanche@ibmcp.upv.es

Keywords: Cell-to-Cell and Systemic Movement, NS_M Protein, NS_M functionality, Tospovirus, Tubule formation, AMV system.

Running title: Functional analysis of NS_M proteins

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.

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, 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 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, 2007; Pappu et al., 2009; Persley, 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.

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*

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™).

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*l, 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.

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*l (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.

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) were amplified with specific primers containing the *Pci*I and *Nhe*I restriction sites. The resultant PCR products were introduced in the construct pSK+35S-eGFP:N-YFP-PoPit (Leastro et al., 2015) by replacing GFP gene using the *Nco*I-*Nhe*I restriction sites. The resultant expression cassette will express the NS_M proteins with the N-terminal 154 aa of the yellow fluorescent protein (Nt-YFP) fused at its C-terminus. For cognate N protein, the C-terminal 84 aa of the YFP (Ct-YFP) was fused at its N-terminal (Leastro et al., 2015). The expression cassettes were subcloned into the $pMOG_{800}$ binary vector. All DNA constructs obtained were confirmed by plasmid DNA sequencing.

2.2. *Inoculation of P12 plants and protoplasts*

Plasmids containing wild-type or the different NS_M mutants were linearized with *Pst*I and transcribed with T7 RNA polymerase (Takara™). Protoplasts were extracted from transgenic *Nicotiana tabaccum* plants that express the polymerase proteins P1 and P2 of AMV (P12 plants; (van Dun et al., 1988) and 2.5 x 10^5 protoplasts were inoculated by the polyethylene glycol method (Loesch-Fries et al., 1985) with the transcription mixture. P12 plants were grown and inoculated with RNA transcripts, as described previously (Taschner et al., 1991). GFP expression in plants was analyzed with a Leica TCS SL confocal laser scanning microscope (Leica), with excitation at 488

nm and emission at 510–560 nm. 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), representing the average of 40 foci from each construction.

2.3. *Northern blot and tissue printing*

Total RNA was extracted from leaves of P12 plants at 15 dpi and from P12 transfected protoplasts at 16 hours after inoculation (hpi). Extraction was performed using Trizol reagent (Invitrogen™) as described previously (Sambrook, 1989). The RNAs were electrophoresed through formaldehyde-denatured gel and transferred to positively charged nylon membranes (Roche Mannheim, Germany).

Tissue-printing analysis were performed by transversal sections of the corresponding petiole from inoculated (I) and upper (U) P12 leaves at 14 dpi, as described previously (Sanchez-Navarro et al., 2010). The nucleic acids were fixed to the nylon membranes using UV cross-linker (700 x100 $\mu J/cm^2$). Hybridization and detection were conducted as described (Pallas et al., 1998) using a dig-riboprobe (Roche Mannheim, Germany) complementary to the AMV 3'UTR.

2.4. *Subcellular localization of the NS_M and N, bimolecular fluorescence complementation assays and Western blot*

To visualize the subcellular localization of BeNMV NS_M and N proteins, the different constructs containing the corresponding protein with the eGFP fused at its C-terminus were transient express in *N. benthamiana*, as reported by Leastro et al., 2015. Callose staining was done as previously described (Leastro et al., 2015).

The BiFC assays were addressed to determine the N-NS_M interactions. *Agrobacterium tumefaciens* (strain C58) cultures (OD_{600}=0,4) transformed with the corresponding binary plasmids $pMOG_{800}$, were used to infiltrate *N. benthamiana* plants as it was previously described (Genovés et al., 2011). To increase the expression of the different proteins that permits a better visualization of the fluorescent signal, both protein were co-expressed with the silencing suppressor HcPro from the *Tobacco each virus*. The plants were kept at 24ºC day-18ºC night, with a 16 h day 8 h night photoperiod. At 4 days post-infiltration, the fluorescence reconstitution was monitored in the confocal Leica TCS SL (λexc=488 nm; λem=500-550 nm).

The accumulation of the transient expression of the N and NS_M proteins was analyzed by Western-blot in 12% SDS-PAGE gels. The gel was electrotransferred to polyvinylidene difluoride membranes following the manufacturer's instructions (Amersham). The detection of the proteins tagged with the CtYFP and NtYFP epitope was performed by using specific antibodies that recognize the N- or C- terminus of the EYFP (Sigma) and a secondary antibody conjugated with the peroxidase (Sigma). The chemiluminescent detection was made using the substrate recommended by Amersham (ECL + Plus Western Blotting Detection System).

3. Results

3.1. *The tospovirus NS_M proteins support differently the cell-to-cell and systemic AMV transport*

First, we evaluated the capability of the different NS_M proteins to support the cell-to-cell and systemic AMV transport. Previous analysis performed with the NS_M of TSWV revealed that this movement protein supports the local and systemic transport of AMV in *N. tabacum* plants that express constitutively the P1 and P2 polymerase proteins of AMV (P12) (Peiro et al., 2014a). To asses that the other tospovirus movement proteins studied in this work support the AMV transport and, particularly, if there are differences in cell-to-cell and systemic AMV movement based on the use of distinct NS_M proteins, the NS_M genes of BeNMV, CSNV and TCSV were exchanged with the corresponding AMV MP gene in the AMV RNA 3 wild-type (wt) (pAL3NcoP3) (van der Vossen et al., 1993) or in a RNA 3 derivative that expresses the GFP (pGFP/A255/CP) (Sanchez-Navarro et al., 2001). In the chimeric constructs, the heterologous NS_M 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) (Sanchez-Navarro et al., 2006). Cell-to-cell movement of the AMV RNA 3 hybrids was studied by inoculation of T7 transcripts into P12 plants (Fig. 1A). All constructs resulted in clear fluorescent infection foci at 2 dpi (Fig. 1A) indicating that the four NS_M proteins were competent to support the local transport of the hybrid AMV RNA 3. The analysis of the area of forty independent foci at 2 and 3 dpi revealed that all foci derived from the chimeric AMV RNA 3 were significantly bigger than the control AMV construct, in which the hybrids RNA 3 carrying the NS_M of BeNMV and TSWV showed the largest areas (Fig. 1B).

The capability of the different tospovirus NS_M to support the systemic transport of the AMV RNA 3 was also 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 plants (Sanchez-Navarro et al., 2001). The accumulation and distribution of the chimeric RNA 3 was then analyzed in inoculated and systemic leaves of P12 plants by tissue printing of petiole cross sections, in which positive hybridization signal correlated with the presence of the virus in the corresponding leaf, as described previously (Fajardo et al., 2013; Mas and Pallas, 1995; Sanchez-Navarro et al., 2010). Results showed that all tospovirus NS_M support the systemic transport of the chimeric AMV RNA 3 (Fig. 1C), in which we observed two different patterns according to the presence of the virus in all (constructs carrying the NS_M gene of TCSV or TSWV) or part (constructs carrying the NS_M gene of CSNV or BeNMV) of the upper not inoculated leaves. Thus, no clear direct correlation was observed between the efficiency on the cell-to-cell and the systemic movement (compare BeNMV vs TSWV in Fig. 1B and 1C).

3.2. *The cell-to-cell movement of tospovirus species is dependent on nucleocapsid assembly*

To examine the strategy used by the tospovirus NS_M to mediate AMV movement, the pGFP/NS_M:A44/CP constructs were further modified to express a shorter CP (CP 206) lacking the C-terminal 14 amino acids. CP 206 was competent for cell-to-cell movement and RNA accumulation but not for virion encapsidation between the viral RNA (Tenllado and Bol, 2000). None of the tospovirus NS_M were able to transport non encapsidated virus, which remained confined to single cells in the presence of CP 206 (Fig. 2A) even though all chimeric AMV constructs were competent to replicate in P12 protoplasts with clear expression of the GFP (Fig. 2B). These results indicate that NS_M proteins analyzed herein are competent to transport only AMV virus particles.

3.3. *NS_M proteins from distinct tospovirus species induce tubular structures in infected P12 protoplasts*

To evaluate possible differences of the property of the NS_M to form tubules of different tospovirus species and to further compare the four NS_M proteins in subsequent assays (deletion assay), we analyzed the polymerization of tubular structures in P12 protoplasts during the AMV infection. To do this, the NS_M genes were introduced in a variant of the AMV RNA 3 (pMP:GFP/CP) that contains the GFP fused at the C-terminus of the viral MP, by exchanging the AMV MP gene (Sanchez-Navarro and Bol, 2001). The derived transcripts were inoculated onto P12 protoplasts and the GFP signal monitored at 16 hours post inoculation. All NS_M proteins induced the formation of tubular structures at the surface of the P12 protoplasts (Fig. 3). However, we observed two different patterns of the tubular fluorescent signal according to the distribution of the fluorescent signal throughout the tube (CSNV, TCSV and TSWV) or discontinuously with a clear signal at the tip of the structure (BeNMV). This discrepancy could reflect tubules formed mainly by the NS_M proteins (uniform GFP signal) or with other cellular molecules (discontinuous GFP signal).

3.4. *The C-terminal region of the BeNMV NS_M is dispensable for cell-to-cell transport*

A common property of the MPs assigned to the ‘30K family’ was the observation that the C-terminal region of the proteins was not necessary for the cell-to-cell transport (Aparicio et al., 2010; Nagano et al., 2001; Sanchez-Navarro and Bol, 2001; Sanchez-Navarro et al., 2006; Takeda et al., 2004). We analyzed if such property could also be applied to the tospovirus NS_M proteins. First we observed that the C-terminal 52 residues of the four NS_M proteins analyzed herein are highly conserved (55%-63% similarity), more relevant between CSNV, TCSV and TSWV with a percentage of similarity that ranged between 93-97%, with the main differences located at the 19 C-terminal residues (Fig. 4). In a second step, we analyzed the requirement of the C-terminus of the NS_M proteins for virus transport by performing different C-terminal deletions and their subsequent functional analysis in the chimeric AMV RNA 3 expressing a free GFP (pGFP/A255/CP). The inoculation of T7 transcripts of the different constructs in P12 plants rendered single infected cells for all CSNV, TCSV and TSWV NS_M mutants, covering deletions from five to seventeen residues of the C-terminus (Fig. 5A), indicating that deletions of five residues is sufficient to make the movement proteins not functional. In the case of the BeNMV NS_M we observed that deletion of 17 residues had no effect on the cell-to-cell transport but increment of 5 residues make the protein not functional, indicating that amino acids located between 295 and 300 of the NS_M, seem to be essential to promote cell-to-cell movement. Further C-terminal deletions covering the last 44 residues rendered the same not-functional NS_M protein (Fig. 5A). We have observed that the C-terminal 17 residues of the BeNMV NS_M protein, representing the more divergent region, compared to the other NS_M proteins, are dispensable for virus transport. Direct comparison between the wild-type BeNMV NS_M ($BeNMV_{wt}$) protein and the mutant lacking the C-terminal 17 residues ($BeNMV_{300}$) revealed that the deletion of the C-terminal region affects the cell-to-cell transport, rendering infection foci with an area representing the 57% of wild-type protein (average of 14 mm^2 vs. 8 mm^2; Fig. 5A). When both proteins were assayed in the wild-type AMV RNA 3 construct, that permits the systemic transport of the chimeric AMV RNA 3, we observed tissue printing hybridization signal in the petioles of the leaves inoculated with both constructs but only hybridization signal in the petiole of the

upper non-inoculated leaves with the construct carrying the wild-type BeNMV NS_M (Fig. 5B). These results indicate that the deletion of the C-terminal 17 residues of the BeNMV NS_M protein negatively affect the cell-to-cell transport and impedes the systemic transport. Finally, we analyzed if the capability of the different C-terminal NS_M mutants that do not support the cell-to-cell transport was correlated with the incapability to generate tubular structures on protoplast. To do this, the NS_M proteins of CSNV, TCSV and TSWV lacking the C-terminal 5 residues ($CSNV_{298}$, $TCSV_{298}$ and $TSWV_{298}$) together with two BeNMV NS_M mutants lacking the 17 ($BeNMV_{300}$) and 22 ($BeNMV_{295}$) residues were assayed in the variant of the AMV RNA 3 described above. All constructs rendered fluorescent dots at the surface of the infected P12 protoplast but not tubular structures, except the construct carrying the $BeNMV_{300}$ NS_M protein that showed shorter tubules when compared with the wild-type construction (Fig. 6). These results indicate that the limitation of the different C-terminal NS_M mutants to support the virus transport was due to the incapability to generate tubular structures, but also that the C-terminal 5 residues of the NS_M proteins of CSNV, TCSV and TSWV or the residues located between the amino acids 295-300 of the BeNMV NS_M are critical to build such structures.

3.5. *The C-terminal 22 residues of the BeNMV NS_M are not required for the interaction with the nucleocapsid protein.*

Previous analysis performed with some MPs of the '30K family' have shown that the C-terminal region of the protein interacts, directly or indirectly, to the cognate CP (Aparicio et al., 2010; Nagano et al., 2001; Sanchez-Navarro and Bol, 2001; Sanchez-Navarro et al., 2006; Takeda et al., 2004). More detailed analysis, have found that the MP motif required for the CP interaction coincided with the C-terminal region dispensable for the cell-to-cell transport (Aparicio et al., 2010; Sanchez-Navarro et al., 2006). In this sense, we decided to check if the dispensable C-terminal motif of the BeNMV NS_M affects the previously reported NS_M-N interaction (Leastro et al., 2015). Before proceeding with the BiFC analysis, we determined the subcellular localization of the BeNMV NS_M and N proteins, in order to assess whether there would be a change in the localization of the proteins upon interaction. The GFP was fused at the C-terminus of both proteins. The fluorescent signal revealed that NS_M protein locates at the cell periphery in different cell aggregates, which correspond to PD (Fig. 7A). In contrast, N protein localized in clusters of different sizes in the cell cytoplasm (Fig. 7A), such as demonstrated by Leastro et al., 2015. In the BiFC assay, the analysis were performed using the wild-type ($BeNMV_{wt}$) and the mutated BeNMV NS_M proteins lacking the C-terminal 17 ($BeNMV_{300}$) or 22 ($BeNMV_{295}$) residues together with the cognate NP, in which the N- (NtYFP) and C-termini (CtYFP) of the yellow fluorescent protein (EYFP) were fused at the C- or the N-termini of the NS_M (NS_M-NtYFP) and the NP (CtYFP-NP) proteins, respectively. This fusion-protein pairs (CtYFP-NP+NS_M-NtYFP) is the best combination that better reproduces the NS_M-N interaction, previously confirmed by BiFC (Dietzgen et al., 2012; Leastro et al., 2015) or by pull down and yeast-2-hybrid assays (Soellick et al., 2000; Tripathi et al., 2015b). All NS_M proteins rendered positive fluorescent signal when co-expressed with the NP, indicating that deletions of the C-terminal 17 or 22 residues do not affect the NS_M-N interaction (Fig. 7B). No fluorescence was observed when both proteins were co-expressed with the corresponding non-fused EYFP fragment or with others N and/or NS_M fusion proteins that do not reconstitute the fluorescent signal (Fig. 7B, panel IV). The transient expression of the all analyzed fusion proteins was confirmed by Western blot analysis (Fig. 7C).

4. Discussion

Viral proteins are intimately related to different host components and minimal alterations of such interactions could have a significant impact in the capability of a virus to infect a determined host (Chen et al., 2008; Ingham and Lazarowitz, 1993; Tatineni et al., 2011). Recent studies have demonstrated the impact of the association of viral proteins with host proteins regulating viral replication and causing changes in the rate of virulence (Nagy, 2015). The viral MPs, responsible to transport the virus to the adjacent cells and/or to the upper leaves, could also play a critical role in the range of susceptible hosts and in the development of the disease (Garcia and Pallas, 2015; Hong et al., 2007; Ingham and Lazarowitz, 1993; Mise and Ahlquist, 1995; Sasaki et al., 2001; Sasaki et al., 2004).

In the present work, we have performed a functional analysis of the MPs (NS_M) of four molecularly and biologically distinct tospoviruses species. Thus, BeNMV has been found from naturally infecting leguminous hosts and has limitation in infecting several botanic families (de Oliveira et al., 2012) meanwhile CSNV, TCSV and TSWV have a broad host-range, including many solanaceae species (de Avila et al., 1993; Pappu et al., 2009). According to these differences, CSNV, TCSV and TSWV have been included in the same clade into the 'New World' tospoviruses, meanwhile BeNMV belongs to a complete new branch of the American species (de Oliveira et al., 2012; Silva et al., 2001). In order to analyze differences in the functionality of the NS_M protein from distinct tospovirus, we took advantage of the AMV model system that permits the functional analysis of MPs assigned to the '30K family', overcoming the absence of tospovirus infectious clones. We observed that the four NS_M proteins analyzed were competent to support the local and the systemic transport of the AMV particles with even more efficient cell-to-cell transport than that observed for the AMV wt. Previous analysis performed with the TSWV NS_M protein also demonstrated that this MP supports the *Tobacco mosaic virus* (Lewandowski and Adkins, 2005) and the AMV transport (Peiro et al., 2014a). The observation that other three NS_M proteins are functionally exchangeable in the AMV system permits the assumption that this must be a general rule for the rest of tospovirus. Regarding the systemic transport, we observed clear differences between the four NS_M proteins analyzed. Thus, the NS_M proteins of TSWV and TCSV allowed the transport of the AMV to all upper non-inoculated leaves meanwhile the NS_M proteins of CSNV and BeNMV supported the systemic transport of AMV to half or a reduced number of the upper leaves, respectively. These results indicate that in spite of the efficient cell-to-cell transport observed for all NS_M proteins, the MPs of CSNV and BeNMV are less efficient for the systemic transport. The majority of tospoviruses cause systemic infection in most of the crop plants they infect (Pappu et al., 2009). In agreement with this, we observed a very efficient systemic transport for the chimeras carrying either the NS_M proteins of TSWV or TCSV. However, CSNV and BeNMV move systemically less frequently, in which the 54% (thirteen out twenty-four host; Bezerra et al., 1999) or the 30% (three out ten hosts; de Oliveira et al., 2012) of the reported hosts are infected systemically, respectively. Taken together, the results reported here point to the idea that the systemic transport of tospoviruses could be conditioned by the NS_M protein. However we cannot rule out the possible involvement of AMV system in the obtained results.

Another question addressed in the present analysis was to see if the four different NS_M proteins differ in the type of viral molecules that are transported between cells. To do this, we used an AMV CP mutant defective in virus formation (CP 206; Tenllado and Bol, 2000), in which positive virus transport indicates that the MP allows virus translocation of viral complexes different to virus particles. We observed that none of the four NS_M proteins were able to transport nonencapsidated viral nucleic acids, which remained confined to single cells in the presence of CP 206. These results are agreed with previous data supporting that tospovirus are moving as a free, non-enveloped ribonucleocapsid structure (Kormelink et al., 1994; Storms et al., 1995). However, contrary results have been reported with the TSWV NS_M protein, which supports the local and systemic transport of a TMV CP deletion mutant (Lewandowski and Adkins, 2005). Apparently, the TSWV NS_M behaves differently in the AMV or TMV viral systems, in which the virus transport is dependent or independent of the virus particles, respectively. If we compare both AMV and TMV systems, we observe that the former requires: i) virus particles for systemic transport in all hosts and ii), the C-terminal modification of the heterologous MP, by fusing the C-terminal 44 amino acids of the AMV MP, for a compatible interaction with the AMV CP. Both processes indicate a direct role of the heterologous MPs in the AMV transport by a MP-CP interaction. In the case of TMV, the observation that the virus could move systemically without the requirement of the CP in some hosts (Knapp et al., 2001; Lewandowski and Adkins, 2005) implies that the virus could move to the upper leaves using other viral complexes different from virus particles. In this sense, it has been described that TMV could invade neighboring cells by the translocation of the replication complexes (Kawakami et al., 2004). The observation that the heterologous TSWV NS_M does not need any modification to be functional in the TMV system (e.g. the presence of the C-termini of the TMV MP to be functional, as observed in the AMV system) opens the possibility that the heterologous MP could support indirectly the TMV transport by building tubular structures between neighboring cells. In this sense, it has been proposed that the TMV transport occurs through the modified plasmodesmata by passive diffusion of the viral complexes (Guenoune-Gelbart et al., 2008), in which the NS_M derived tubular structures could be sufficient to support the TMV transport.

The incapability observed for the tospovirus NS_M proteins to facilitate the spread of vRNA complexes in the AMV context, has been only observed for the 30K MPs of DNA viruses (Sanchez-Navarro et al., 2010). Meanwhile, all analyzed MPs of the '30K family' from RNA viruses were competent to transport nucleoprotein complexes - NPC (Sanchez-Navarro et al., 2006), independently of the capability of the MP to form tubular structures. The differences observed between the MPs of DNA and RNA viruses were hypothesized to be a consequence of the restriction of the endogenous cell-to-cell transport via PD that is specialized to use RNA as the communication and signaling molecule (Lucas et al., 2001). Thus, the transport of virus particles ensures safe transport of the viral DNA genome through the non-cell-autonomous RNA-specific pathway (Sanchez-Navarro et al., 2010). However, the results presented herein open the question why the tospovirus NS_M are not competent to transport vRNA complexes in spite of being RNA viruses? A possible explanation could be related to the idiosyncrasy of tospovirus infecting plant and insect organisms. In addition to virus particle, the capability to transport NPC between cells has been reported only for plant viruses meanwhile virions is a prerequisite for animal or insect virus transport (Bilkova et al., 2014; Kilcher and Mercer, 2015; Marsh and Helenius, 2006). In this sense, we can hypothesize that the NS_M of tospovirus have evolved to transport only nucleoprotein complexes that is compatible both plant and insect organism. The NPC transport by the

NS_M proteins implies a N-NS_M interaction; such interaction has been reported by pull down experiments (Soellick et al., 2000) and more recently, by transient expression of both proteins (Leastro et al., 2015; Tripathi et al., 2015b).

The four NS_M proteins analyzed herein induce the formation of tubular structures on the surface of infected P12 protoplasts, suggesting that such ability could be a general property of the Tospovirus genus. We observed that C-terminal NS_M deletions rendered two different patterns: NS_M proteins (CSNV, TCSV and TSWV) in which deletions of 5 residues make the protein non-functional and the NS_M of BeNMV that permits the deletion of 17 residues. In all cases, the lack of function of the NS_M always correlated with the incapability of the protein to generate tubular structures as reported for different MPs of the '30K family', including the TSWV (Aparicio et al., 2010; Li et al., 2009; Sanchez-Navarro and Bol, 2001). However, different results have been reported for the NS_M of TSWV using the TMV viral systems, in which deletion of the C-terminal 54 residues rendered a protein incapable to generate tubular structures but still functional, with a reduced cell-to-cell transport sufficient to form tiny local lesions (Lewandowski and Adkins, 2005). The C-terminal region of several MPs of the '30K family' has been shown to interact with the cognate CP (Aparicio et al., 2010; Berna et al., 1991; Carvalho et al., 2003; Kim et al., 2004; Nagano et al., 1997; Sanchez-Navarro and Bol, 2001; Takeda et al., 2004). However, we observed a completely different situation for the NS_M proteins since the C-terminus is required for the functionality of the protein (CSNV, TCSV and TSWV) and is not required (BeNMV, herein; *Iris yellow spot virus*, Tripathi et al., 2015a) for an interaction with the N protein. Previous results have reported that the NS_M of as *Groundnut bud necrosis virus* associates with membranes via the C-terminal coiled-coil domain, which is also required for the functionality of the protein (Singh et al., 2014). The four NS_M proteins analyzed have a predicted C-terminal coiled-coil domain (Leastro et al., 2015), but differ in the regions required for such structure. Thus, meanwhile this region covers the majority of the C-terminus of the NS_M proteins of CSNV, TCSV and TSWV (until residue 297-299 out 303-302) in the case of the BeNMV NS_M, this region includes until residue 296 out of 317. Interestingly, our C-terminal deletion results are compatible with the predicted coiled-coil domain of the four NS_M proteins, suggesting that any deletion that could affect this structure make the protein non-functional. In this sense, deletion of the left border but not in the central part of the coiled-coil domain of the TSWV NS_M protein rendered non-functional protein (Li et al., 2009). In this scenario, the open question is to know what could be the putative function of the dispensable C-terminal residues of the BeNMV NS_M. The observation that the mutated $BeNMV_{300}$ NS_M protein generates shorter tubular structures than the wild-type protein and a reduced cell-to-cell transport open the possibility that this region could be stabilizing the coiled coil domain, although further analysis are required to confirm this hypothesis.

5. Conclusions

The *in vivo* results showed common properties but also clear differences between the NS_M proteins of tospovirus species molecularly and biologically distinct. In the AMV system, all NS_M proteins transport virus particles, required the formation of tubular structures and possess a C-terminal region with different functions of other MPs of the '30K family'. We observed that the NS_M of BeNMV, with a very limited range of hosts and phylogenetically more distant, is very efficient for cell-to-cell transport but very inefficient for the systemic infection, with a C-terminal region that is dispensable

for virus transport. These results also indicate a possible different role and/or transport mechanism promoting short and long distance tospovirus movement. Overall, these differences seem to reveal a distinct tospovirus evolution and/or host adaption that can correlate with their distinct efficiency to promote the cell-to-cell and systemic movement within the host species.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgements

We thank L. Corachán for her excellent technical assistance. This work was supported by grant BIO2014-54862-R from the Spanish Direccion General de Investigacion Cientifica y Tecnica (DGICYT), the Prometeo Program GV2014/010 from the Generalitat Valenciana, CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Capes (Conselho de Aperfeiçoamento de Pessoal de Nível Superior) and FAP-DF (Fundação de Apoio a Pesquisa do Distrito Federal).

References

Adkins, S., 2000. Tomato spotted wilt virus-positive steps towards negative success. Mol. Plant Pathol. 1 (3), 151-157.

Aparicio, F., Pallas, V., Sanchez-Navarro, J., 2010. Implication of the C terminus of the Prunus necrotic ringspot virus movement protein in cell-to-cell transport and in its interaction with the coat protein. J. Gen. Virol. 91 (7), 1865-1870.

Benitez-Alfonso, Y., Faulkner, C., Ritzenthaler, C., Maule, A.J., 2010. Plasmodesmata: gateways to local and systemic virus infection. Mol. Plant-Microbe Interact. 23 (11), 1403-1412.

Berna, A., Gafny, R., Wolf, S., Lucas, W.J., Holt, C.A., Beachy, R.N., 1991. The TMV movement protein: role of the C-terminal 73 amino acids in subcellular localization and function. Virology 182 (2), 682-689.

Bezerra, I.C., de, O.R.R., Pozzer, L., Nagata, T., Kormelink, R., De Avila, A.C., 1999. Increase of tospoviral diversity in Brazil with the identification of two new tospovirus species, one from chrysanthemum and one from zucchini. Phytopathol. 89 (9), 823-830.

Bilkova, E., Forstova, J., Abrahamyan, L., 2014. Coat as a dagger: the use of capsid proteins to perforate membranes during non-enveloped DNA viruses trafficking. Viruses 6 (7), 2899-2937.

Carrington, J.C., Kasschau, K.D., Mahajan, S.K., Schaad, M.C., 1996. Cell-to-Cell and Long-Distance Transport of Viruses in Plants. The Plant cell 8 (10), 1669-1681.

Carvalho, C.M., Wellink, J., Ribeiro, S.G., Goldbach, R.W., Van Lent, J.W., 2003. The C-terminal region of the movement protein of Cowpea mosaic virus is involved in binding to the large but not to the small coat protein. J. Gen. Virol. 84 (8), 2271-2277.

Chen, K.C., Chiang, C.H., Raja, J.A., Liu, F.L., Tai, C.H., Yeh, S.D., 2008. A single amino acid of niapro of papaya ringspot virus determines host specificity for infection of papaya. Mol. Plant-Microbe Interact. 21 (8), 1046-1057.

de Avila, A.C., de Haan, P., Kormelink, R., Resende Rde, O., Goldbach, R.W., Peters, D., 1993. Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. J. Gen. Virol. 74 (2), 153-159.

de Haan, P., Kormelink, R., de Oliveira Resende, R., van Poelwijk, F., Peters, D., Goldbach, R., 1991. Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J. Gen. Virol. 72 (9), 2207-2216.

de Oliveira, A.S., Melo, F.L., Inoue-Nagata, A.K., Nagata, T., Kitajima, E.W., Resende, R.O., 2012. Characterization of bean necrotic mosaic virus: a member of a novel evolutionary lineage within the Genus Tospovirus. PloS one 7 (6), e38634.

Dietzgen, R.G., Martin, K.M., Anderson, G., Goodin, M.M., 2012. In planta localization and interactions of impatiens necrotic spot tospovirus proteins. J. Gen. Virol. 93 (11), 2490-2495.

Fajardo, T.V., Peiro, A., Pallas, V., Sanchez-Navarro, J., 2013. Systemic transport of Alfalfa mosaic virus can be mediated by the movement proteins of several viruses assigned to five genera of the 30K family. J. Gen. Virol. 94 (3), 677-681.

Fernandez-Calvino, L., Faulkner, C., Walshaw, J., Saalbach, G., Bayer, E., Benitez-Alfonso, Y., Maule, A., 2011. Arabidopsis plasmodesmal proteome. PloS one 6(4), e18880.

Garcia, J.A., Pallas, V., 2015. Viral factors involved in plant pathogenesis. Curr. Opin. Virol. 11, 21-30.

Genovés, A., Pallás, V., Navarro, J.A., 2011. Contribution of topology determinants of a viral movement protein to its membrane association, intracellular traffic, and viral cell-to-cell movement. J. Virol. 85 (15), 7797-7809.

Guenoune-Gelbart, D., Elbaum, M., Sagi, G., Levy, A., Epel, B.L., 2008. Tobacco mosaic virus (TMV) replicase and movement protein function synergistically in facilitating TMV spread by lateral diffusion in the plasmodesmal desmotubule of Nicotiana benthamiana. Mol. Plant-Microbe Interact. 21 (3), 335-345.

Hallwass, M., de Oliveira, A.S., de Campos Dianese, E., Lohuis, D., Boiteux, L.S., Inoue-Nagata, A.K., Resende, R.O., Kormelink, R., 2014. The Tomato spotted wilt virus cell-to-cell movement protein (NSM) triggers a hypersensitive response in Sw-5-containing resistant tomato lines and in Nicotiana benthamiana transformed with the functional Sw-5b resistance gene copy. Mol. Plant Pathol. 15 (9), 871-880.

Heinlein, M., Epel, B.L., 2004. Macromolecular transport and signaling through plasmodesmata. Int. Rev. Cytol. 235, 93-164.

Hong, J.S., Ohnishi, S., Masuta, C., Choi, J.K., Ryu, K.H., 2007. Infection of soybean by cucumber mosaic virus as determined by viral movement protein. Arch. Virol. 152 (2), 321-328.

Ingham, D.J., Lazarowitz, S.G., 1993. A single missense mutation in the BR1 movement protein alters the host range of the squash leaf curl geminivirus. Virology 196 (2), 694-702.

Kawakami, S., Watanabe, Y., Beachy, R.N., 2004. Tobacco mosaic virus infection spreads cell to cell as intact replication complexes. Proc. Natl. Acad. Sci. USA 101 (16), 6291-6296.

Kilcher, S., Mercer, J., 2015. DNA virus uncoating. Virology 479-480, 578-590.

Kim, S.H., Kalinina, N.O., Andreev, I., Ryabov, E.V., Fitzgerald, A.G., Taliansky, M.E., Palukaitis, P., 2004. The C-terminal 33 amino acids of the cucumber mosaic virus 3a protein affect virus movement, RNA binding and inhibition of infection and translation. J. Gen. Virol. 85 (1), 221-230.

Knapp, E., Dawson, W.O., Lewandowski, D.J., 2001. Conundrum of the lack of defective RNAs (dRNAs) associated with tobamovirus Infections: dRNAs that can move are not replicated by the wild-type virus; dRNAs that are replicated by the wild-type virus do not move. J. Virol. 75 (12), 5518-5525.

Kormelink, R., Storms, M., Van Lent, J., Peters, D., Goldbach, R., 1994. Expression and subcellular location of the NSM protein of tomato spotted wilt virus (TSWV), a putative viral movement protein. Virology 200 (1), 56-65.

Lazarowitz, S.G., 1999. Probing plant cell structure and function with viral movement proteins. Curr. Opin. Plant Biol. 2 (4), 332-338.

Lazarowitz, S.G., Beachy, R.N., 1999. Viral movement proteins as probes for intracellular and intercellular trafficking in plants. The Plant cell 11 (4), 535-548.

Leastro, M.O., Pallas, V., Resende, R.O., Sanchez-Navarro, J.A., 2015. The movement proteins (NSm) of distinct tospoviruses peripherally associate with cellular membranes and interact with homologous and heterologous NSm and nucleocapsid proteins. Virology 478, 39-49.

Lebas, B.S.M., Ochoa-Corona, F.M., 2007. Impatiens necrotic spot virus. In: Rao, G.P., Bragard, C., Lebas, B.S.M. (Eds.), Characterization, Diagnosis and Management of Plant Viruses. Grain Crops and Ornamentals. Stadium Press LLC, Texas, 4, 221–243.

Lewandowski, D.J., Adkins, S., 2005. The tubule-forming NSm protein from Tomato spotted wilt virus complements cell-to-cell and long-distance movement of Tobacco mosaic virus hybrids. Virology 342 (1), 26-37.

Li, W., Lewandowski, D.J., Hilf, M.E., Adkins, S., 2009. Identification of domains of the Tomato spotted wilt virus NSm protein involved in tubule formation, movement and symptomatology. Virology 390 (1), 110-121.

Loesch-Fries, L.S., Jarvis, N.P., Krahn, K.J., Nelson, S.E., Hall, T.C., 1985. Expression of alfalfa mosaic virus RNA 4 cDNA transcripts in vitro and in vivo. Virology 146 (2), 177-187.

Lucas, W.J., 2006. Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. Virology 344 (1), 169-184.

Lucas, W.J., Yoo, B.C., Kragler, F., 2001. RNA as a long-distance information macromolecule in plants. Nature reviews. Molecular cell biology 2(11), 849-857.

Marsh, M., Helenius, A., 2006. Virus entry: open sesame. Cell 124 (4), 729-740.

Mas, P., Pallas, V., 1995. Non-isotopic tissue-printing hybridization: a new technique to study long-distance plant virus movement. J. Virol. Methods 52 (3), 317-326.

Melcher, U., 2000. The '30K' superfamily of viral movement proteins. J. Gen. Virol. 81(1), 257-266.

Mise, K., Ahlquist, P., 1995. Host-specificity restriction by bromovirus cell-to-cell movement protein occurs after initial cell-to-cell spread of infection in nonhost plants. Virology 206 (1), 276-286.

Mushegian, A.R., Elena, S.F., 2015. Evolution of plant virus movement proteins from the 30K superfamily and of their homologs integrated in plant genomes. Virology 476, 304-315.

Nagano, H., Mise, K., Furusawa, I., Okuno, T., 2001. Conversion in the requirement of coat protein in cell-to-cell movement mediated by the cucumber mosaic virus movement protein. J. Virol. 75 (17), 8045-8053.

Nagano, H., Okuno, T., Mise, K., Furusawa, I., 1997. Deletion of the C-terminal 33 amino acids of cucumber mosaic virus movement protein enables a chimeric brome mosaic virus to move from cell to cell. J. Virol. 71 (3), 2270-2276.

Nagy, P.D., 2015. Viral sensing of the subcellular environment regulates the assembly of new viral replicase complexes during the course of infection. J. Virol. 89 (10), 5196-5199.

Niehl, A., Heinlein, M., 2011. Cellular pathways for viral transport through plasmodesmata. Protoplasma 248 (1), 75-99.

Pallas, V., Garcia, J.A., 2011. How do plant viruses induce disease? Interactions and interference with host components. J. Gen. Virol. 92 (12), 2691-2705.

Pallas, V., Mas, P., Sanchez-Navarro, J.A., 1998. Detection of plant RNA viruses by nonisotopic dot-blot hybridization. Methods Mol. Biol. 81, 461-468.

Pappu, H.R., Jones, R.A., Jain, R.K., 2009. Global status of tospovirus epidemics in diverse cropping systems: successes achieved and challenges ahead. Virus Res. 141 (2), 219-236.

Peiro, A., Canizares, M.C., Rubio, L., Lopez, C., Moriones, E., Aramburu, J., Sanchez Navarro, J., 2014a. The movement protein (NSm) of Tomato spotted wilt virus is the avirulence determinant in the tomato Sw-5 gene-based resistance. Mol. Plant Pathol. 15 (8), 802-813.

Peiro, A., Martinez-Gil, L., Tamborero, S., Pallas, V., Sanchez-Navarro, J.A., Mingarro, I., 2014b. The Tobacco mosaic virus movement protein associates with but does not integrate into biological membranes. J. Virol. 88 (5), 3016-3026.

Persley, D.M., Thomas, J.E., Sharman, M., 2006. Tospoviruses—an Australian perspective. Australas. Plant Pathol. 35, 161–180.

Ribeiro, D., Borst, J.W., Goldbach, R., Kormelink, R., 2009. Tomato spotted wilt virus nucleocapsid protein interacts with both viral glycoproteins Gn and Gc in planta. Virology 383 (1), 121-130.

Ritzenthaler, C., Hofmann, C., 2007. Tubule-guided movement of plant viruses. In: Viral transport in plants. Eds. E. Waigmann and M. Heinlein. Springer-Verlag Berlin, 63-83.

Sambrook, J., Fritsch, E.F., Manuatis, T., 1989. Molecular Cloning: a laboratory manual. . N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press (ISBN 0-87969-309-6.), 1989-1659

Sanchez-Navarro, J., Fajardo, T., Zicca, S., Pallas, V., Stavolone, L., 2010. Caulimoviridae tubule-guided transport is dictated by movement protein properties. J. Virol. 84 (8), 4109-4112.

Sanchez-Navarro, J., Miglino, R., Ragozzino, A., Bol, J.F., 2001. Engineering of alfalfa mosaic virus RNA 3 into an expression vector. Arch. Virol. 146 (5), 923-939.

Sanchez-Navarro, J.A., Bol, J.F., 2001. Role of the alfalfa mosaic virus movement protein and coat protein in virus transport Mol. Plant-Microbe Interact. 14 (9), 1051-1062.

Sanchez-Navarro, J.A., Carmen Herranz, M., Pallas, V., 2006. Cell-to-cell movement of Alfalfa mosaic virus can be mediated by the movement proteins of Ilar-, bromo-, cucumo-, tobamo- and comoviruses and does not require virion formation. Virology 346 (1), 66-73.

Sasaki, N., Fujita, Y., Mise, K., Furusawa, I., 2001. Site-specific single amino acid changes to Lys or Arg in the central region of the movement protein of a hybrid bromovirus are required for adaptation to a nonhost. Virology 279 (1), 47-57.

Sasaki, N., Kaido, M., Okuno, T., Mise, K., 2004. Effects of artificial codon changes in the movement protein gene on adaptation of a hybrid bromovirus to cowpea. Microbiol. Immunol. 48 (2), 131-135.

Scholthof, H.B., 2005. Plant virus transport: motions of functional equivalence. Trends Plant Sci. 10 (8), 376-382.

Silva, M.S., Martins, C.R., Bezerra, I.C., Nagata, T., de Avila, A.C., Resende, R.O., 2001. Sequence diversity of NS(M) movement protein of tospoviruses. Arch. Virol. 146 (7), 1267-1281.

Sin, S.H., McNulty, B.C., Kennedy, G.G., Moyer, J.W., 2005. Viral genetic determinants for thrips transmission of Tomato spotted wilt virus. Proc. Natl. Acad. Sci. USA 102 (14), 5168-5173.

Singh, P., Indi, S.S., Savithri, H.S., 2014. Groundnut bud necrosis virus encoded NSm associates with membranes via its C-terminal domain. PloS one 9 (6), e99370.

Soellick, T., Uhrig, J.F., Bucher, G.L., Kellmann, J.W., Schreier, P.H., 2000. The movement protein NSm of tomato spotted wilt tospovirus (TSWV): RNA binding, interaction with the TSWV N protein, and identification of interacting plant proteins. Proc. Natl. Acad. Sci. USA 97 (5), 2373-2378.

Storms, M.M., Kormelink, R., Peters, D., Van Lent, J.W., Goldbach, R.W., 1995. The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology 214 (2), 485-493.

Takeda, A., Kaido, M., Okuno, T., Mise, K., 2004. The C terminus of the movement protein of Brome mosaic virus controls the requirement for coat protein in cell-to-cell movement and plays a role in long-distance movement. J. Gen. Virol. 85 (6), 1751-1761.

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S., Okuno, T., 2002. Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. FEBS Lett. 532 (1-2), 75-79.

Taschner, P.E., van der Kuyl, A.C., Neeleman, L., Bol, J.F., 1991. Replication of an incomplete alfalfa mosaic virus genome in plants transformed with viral replicase genes. Virology 181 (2), 445-450.

Tatineni, S., Robertson, C.J., Garnsey, S.M., Dawson, W.O., 2011. A plant virus evolved by acquiring multiple nonconserved genes to extend its host range. Proc. Natl. Acad. Sci. USA 108 (42), 17366-17371.

Tenllado, F., Bol, J.F., 2000. Genetic dissection of the multiple functions of alfalfa mosaic virus coat protein in viral RNA replication, encapsidation, and movement. Virology 268 (1), 29-40.

Tripathi, D., Raikhy, G., Goodin, M.M., Dietzgen, R.G., Pappu, H.R., 2015a. In Vivo Localization of Iris yellow spot Tospovirus (Bunyaviridae)-Encoded Proteins and Identification of Interacting Regions of Nucleocapsid and Movement Proteins. PloS one 10 (3), e0118973.

Tripathi, D., Raikhy, G., Pappu, H.R., 2015b. Movement and nucleocapsid proteins coded by two tospovirus species interact through multiple binding regions in mixed infections. Virology 478, 143-153.

Ueki, S.C., V., 2007. Spread throughout the plant: systemic transport of viruses. Plant Cell Monogr. 7, 85-118.

van der Vossen, E.A., Neeleman, L., Bol, J.F., 1993. Role of the 5' leader sequence of alfalfa mosaic virus RNA 3 in replication and translation of the viral RNA. Nucleic Acids Res. 21 (6), 1361-1367.

van Dun, C.M., van Vloten-Doting, L., Bol, J.F., 1988. Expression of alfalfa mosaic virus cDNA1 and 2 in transgenic tobacco plants. Virology 163 (2), 572-578.

Waigmann, E., Ueki, S., Trutnyeva, K., Citovsky, V., 2004. The ins and outs of non-destructive cell-to-cell and systemic movement of plant viruses. Crit. Rev. Plant Sci. 23, 195-250.

Wolf, S., Deom, C.M., Beachy, R.N., Lucas, W.J., 1989. Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science 246 (4928), 377-379.

Zhou, J., Kantartzi, S.K., Wen, R.H., Newman, M., Hajimorad, M.R., Rupe, J.C., Tzanetakis, I.E., 2011. Molecular characterization of a new Tospovirus infecting soybean. Virus genes 43 (2), 289-295.

Figure legends

Fig. 1. Analysis of the cell-to-cell and systemic transport of the hybrid AMV (*Alfalfa mosaic virus*-AMV) RNA 3 in which its movement protein (MP) gene was exchanged with the corresponding genes (NS_M) of the viruses: *Bean necrotic mosaic virus* (BeNMV), *Chrysanthemum stem necrosis virus* (CSNV), *Tomato chlorotic spot virus* (TCSV), *Tomato spotted wilt virus* (TSWV) and AMV. **(A)** Infection foci observed in P12 plants inoculated with RNA 3 transcripts from pGFP/A255/CP derivatives carrying the NS_M genes of BeNMV, CSNV, TCSV, TSWV and AMV MPs. The schematic representation shows the GFP/A255/CP AMV RNA 3, 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. Checkered box corresponding to the C-terminal 44 amino acids of the AMV MP meanwhile arrows represent subgenomic promoters. Blue numbers represent the total amino acids residues of the corresponding MP. The *Nco*I and *Nhe*I restriction sites used for insertions of the MPs are indicate, as well as the restriction sites *BspH*I, *Pci*I and *Nhe*I. Images contain representative infection foci of *Nicotiana tabacum* P12 tobacco plants infected with the chimerical constructs expressing the GFP. White bars corresponding at 200 µm. **(B)** Histograms represent the average of the area in mm^2 of 40 independent infection foci at 2 and 3 days post inoculation (dpi), which error bars indicate the standard deviation. **(C)** Tissue printing analysis of P12 plants inoculated with the AMV RNA 3 derivatives showed in A but lacking the 5' proximal GFP gene. 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. '-' refers to non-inoculated plant.

Fig. 2. Analysis of cell-to-cell transport of the hybrid AMV RNA3, carrying the different NS_M genes and the mutated coat protein gene (CP-N206) defective in virus particles formation. **(A)** The schematic representation correspond to the same constructs indicated in Fig. 1A, in which the CP gene was replaced by the CP-N206 gene from the construct pGFP/BMV:A44/CP-N206 (Sanchez-Navarro et al., 2006) by exchanging the *Nhe*I-*Pst*I fragment. P12 plants or protoplast were inoculated with transcripts derived from the AMV RNA 3 variants. Images correspond to representative pictures of the infected cells observed at 1 (P12 protoplasts) or 2 (P12 plants) days post-inoculation (dpi) using a Leica Stereoscopic Microscope. Long and short bars represent 500 and 50 µm, respectively. **(B)** Northern blot analysis of the accumulation of the chimeric AMV RNAs in P12 protoplasts inoculated with RNA transcribed from the constructs shown in (A). The position of the chimeric RNA 3 and 4 and additional subgenomic RNA

(sgRNA) are indicated on the left margin. rRNA indicates 23S RNA loading control. '-' refers to mock inoculated plant.

Fig. 3. Analysis of tubule formation of the NS_M proteins on *Nicotiana tabacum* P12 protoplasts. The schematic representation shows the construct pMP:GFP/CP (Sanchez-Navarro et al., 2001) 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. Arrows indicates the subgenomic promoter. Single boxes represent the NS_M genes of BeNMV, CSNV, TCSV and TSWV exchanged by the MP of AMV. The restriction sites used for the exchange of the AMV MP gene with the corresponding NS_M genes are indicated. Blue numbers represent the total amino acids residues of the corresponding NS_M. The assay was performed three times from P12 protoplast transfected with RNA 3 hybrid transcripts. Each image-frame expressing GFP represents the visualization of several protoplasts (about 15 to 20) per assay for each NS_M protein analyzed. The fluorescence was monitored after 16 hours using a confocal microscopy Leica TCS SL. Images correspond to the tubular structures observed for the NS_M of BeNMV (I), CSNV (II), TCSV (II) and TSWV (IV). From left to right, GFP fluorescence, brightfield and overlapping images are shown. White bars correspond to 50 µm.

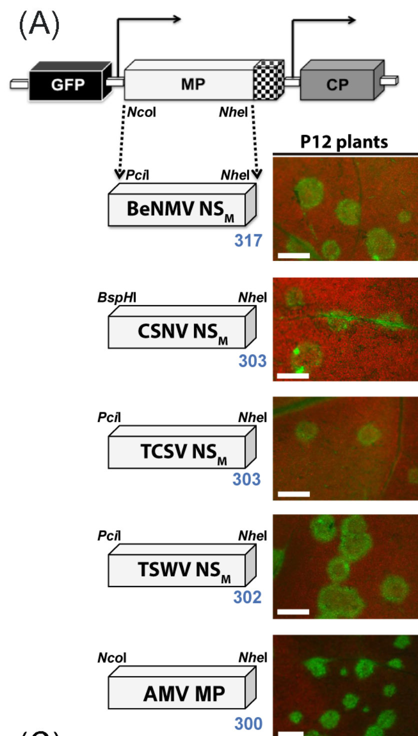
Fig. 4. Alignment of the C-terminus of the NS_M proteins of BeNMV, CSNV, TCSV and TSWV. Consensus sequence among the NS_M proteins is shown. The numbers on the right indicate the total size of the NS_M proteins. Red underlined residues indicate the regions with high values for coiled coil domains identified by coils prediction server (http://toolkit.tuebingen.mpg.de/pcoils). The green and the blue boxes indicate the last residue of the large and the small C-terminal deletions, respectively.

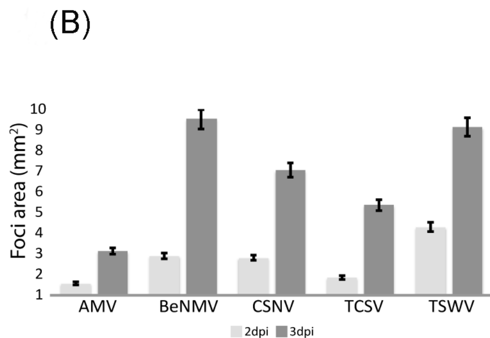
Fig. 5. Truncation assay of the tospovirus NS_M to identify C-terminal residues dispensable for virus transport. **(A)** Infection foci observed in P12 plants inoculated with RNA 3 transcripts from pGFP/A255/CP derivatives carrying the C-terminal truncated NS_M genes of BeNMV, CSNV, TCSV and TSWV. The Schematic representation shows the chimeric GFP/A255/CP AMV RNA 3 represented in Fig. 1A and the C-terminal deletions of the NS_M proteins analyzed. Amino acid numbers 302, 303 and 317 correspond to wild-type (wt) size of the corresponding NS_M proteins meanwhile residues numbers 273, 276, 279, 282, 285, 288, 292, 295 and 300 correspond to the last amino acid of the BeNMV NS_M protein included in the corresponding truncation. Amino acids 286, 289, 292, 295, and 298 correspond to the last residue of the NS_M of CSNV, TCSV and TSWV included in the corresponding truncation. Dashed lines represent the amino acids deleted from the C-terminus of the NS_M proteins. Images contain single infected cells or infection foci of P12 tobacco plants infected with the chimerical constructs expressing the GFP at 2 dpi. White bars correspond to 200 µm. Histograms represent the average of the area in mm^2 of 40 independent infection foci at 2 and 3 dpi of the P12 plant infected with hybrids AMV RNA 3 containing the BeNMV NS_M wild-type or lacking the C-terminal 17 residues ($BeNMV_{300}$). Error bars indicate the standard deviation. **(B)** Tissue printing analysis of P12 plants inoculated with the AMV RNA 3 derivatives showed in A but lacking the 5' proximal GFP gene and containing the AMV MP (AMV), the $BeNMV_{wt}$ or $BeNMV_{300}$. 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 that correspond to the position of the leaves in the plant from the lower to

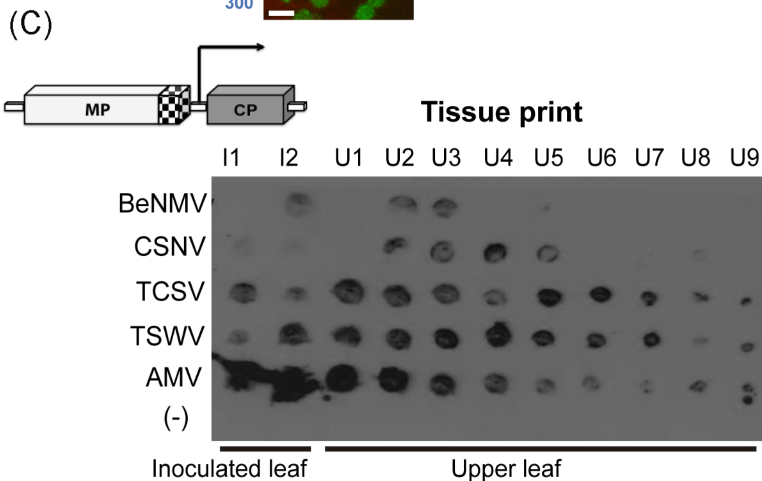
the upper part in which U1 corresponds to the closest one to the inoculated leaf. '-' refers to non-inoculated plant.

Fig. 6. Analysis of tubule formation of the truncated NS_M proteins of the BeNMV, CSNV, TCSV and TSWV on *Nicotiana tabacum* P12 protoplasts. The schematic representation shows the construct pMP:GFP/CP represented in Fig. 3, containing the truncated NS_M genes that express the N-terminal 295 ($BeNMV_{295}$), 300 ($BeNMV_{300}$) or 298 ($CSNV_{298}$; $TCSV_{298}$; $TSWV_{298}$) residues. P12 protoplasts were transfected with RNA 3 hybrid transcripts and the fluorescence monitored after 16 hours using a confocal microscopy Leica TCS SL. From left to right, GFP fluorescence and bright fields with overlapping images are shown. White bars correspond to 50 µm.

Fig. 7. BiFC analysis of the interaction between truncated movement proteins (NS_M) and the nucleocapsid protein (N) of BeNMV. (A) Subcellular location of NS_M and N protein, carrying GFP (•) fused at their C-terminus. In the case of the NS_M protein, the callose deposit of the infiltrated *N. benthamiana* leaves were stained using Analine blue (•). The NS_M protein and the fluorochrome accumulate in similar punctate patterns at cell periphery suggesting that the NS_M protein accumulate in PDs. Thick and thin bars correspond to 50 µm and 10 µm **(B)** The NS_M and/or N proteins, carrying the N-terminal (•) or the C-terminal (•) EYFP fragments fused at their N- (•-N) or C-terminus (NS_M-•), were transiently co-expressed in *N. benthamiana* leaves by agroinfiltration. Confocal microscopy images correspond to the fluorescence reconstitution monitored during the co-expression of the N protein with the wild-type (I) or the truncated NS_M proteins expressing the N-terminal 295 (II) or 300 (III) residues, at 4 days post-infiltration. All images contain two pictures corresponding to the YFP fluorescence alone or plus the brightfield. Negative controls correspond to the expression of the NS_M and N proteins in combination with N-terminal (•) EYFP fragment or with different fusion protein pair combinations (IV). White bars correspond to 50 µm. **(C)** Western immunoblot analysis of the transient accumulation of the N and NS_M proteins in *N. benthamiana* leaves. Monoclonal antibodies that recognizes the N- (anti-•) or C- (anti-•) terminus of the EYFP (Sigma) were used to detect the N and NS_M proteins fused to the Ct-YFP and Nt-YFP fragments. Numbers in parenthesis indicate the molecular weight in kDa of the corresponding fusion protein. a, b, c, d and e represent the different negative control combinations showed in B.


(A)
GFP
MP
CP
NcoI
NheI
P12 plants
PciI
NheI
BeNMV NSM
317
BspHI
NheI
CSNV NSM
303
PciI
NheI
TCSV NSM
303
PciI
NheI
TSWV NSM
302
NcoI
NheI
AMV MP
300


(B)
Foci area (mm2)
1
2
3
4
5
6
7
8
9
10
AMV
BeNMV
CSNV
TCSV
TSWV
2dpi
3dpi


(C)
MP
CP
Tissue print
I1
I2
U1
U2
U3
U4
U5
U6
U7
U8
U9
BeNMV
CSNV
TCSV
TSWV
AMV
(-)
Inoculated leaf
Upper leaf

(A)

GFP MP CP206

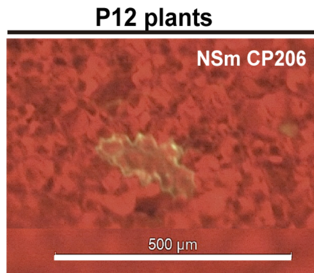
NcoI NheI

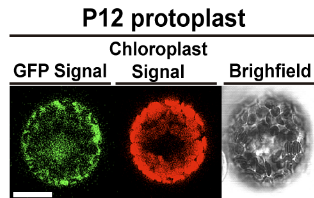
PciI NheI
BeNMV NS_M
317

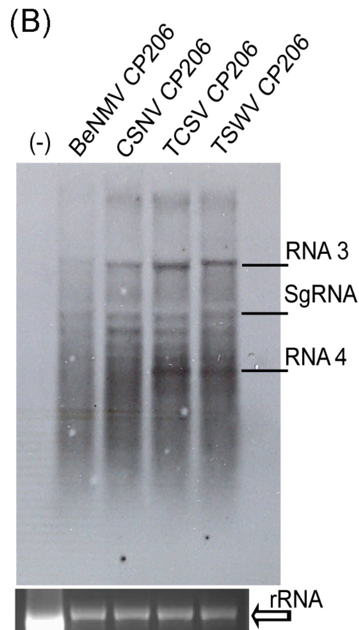
BspHI NheI
CSNV NS_M
303

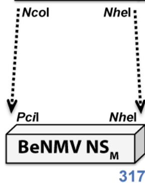
PciI NheI
TCSV NS_M
303

PciI NheI
TSWV NS_M
302

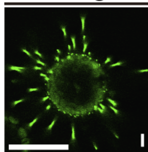


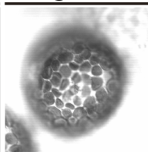


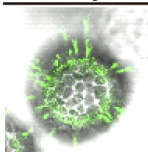


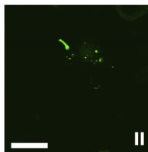


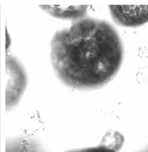
GFP Signal | Brightfield | Overlay

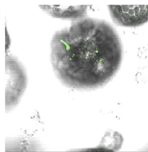


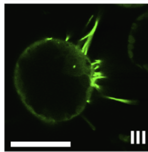


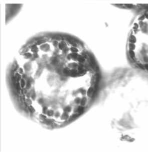


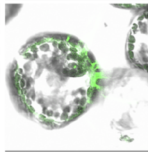


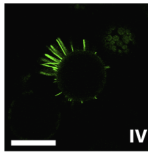


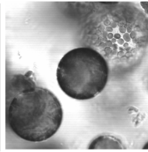


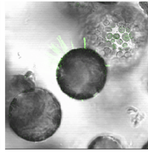












```
                 270                273       280                   290
Consensus        K L I   I P K G N S E K Q I X K Q L K   D L S S N L E R
1. BeNMV NSm     K Y M   I P K G S S G K A I R K Q I E   S L G K H L E Q
2. CSNV NSm      K L I   I P K G N S E K Q I R K Q L K   D L S S N L E R
3. TCSV NSm      K L I   I P K G N S A K Q I K K Q L K   D L S T N L E K
4. TSWV NSm      K L I   I P K G N S E K Q I K K Q L K   E L S S N L E R

                       286      300  300                310                 319
Consensus        S V E E E E E   G V S D N I A K L S F X D E I   * M T *
1. BeNMV NSm     A A I D E E N   E E V S N E T E S S F P P L K   L M T * 317
2. CSNV NSm      S V E E E E E   G V S E N I A K L S F I D E V   * 303
3. TCSV NSm      S V E E E E E   G V C D N I A K L S F V D E I   * 303
4. TSWV NSm      S V E E E E E   G I S D S V A Q L S F - D E I   * 302
                                                 298
```

(A)

GFP
MP
CP

N-terminal
BeNMV NS_M
C-terminal

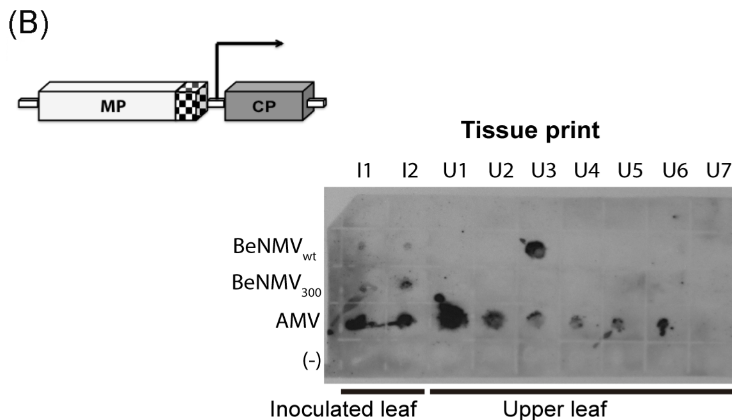
ATG
317
300 17 a.a
295 22 a.a
292
288
285
282
279
276 41 a.a
273 44 a.a

CSNV, TCSV, and TSWV NS_M

ATG
303
298
5 a.a
295
292
289
286

P12 Plants

16
14
12
10
8
6
4
2
0
Foci Area (mm^2)
2dpi
3dpi
$BeNMV_{300}$ $BeNMV_{wt}$



GFP Signal | Overlay

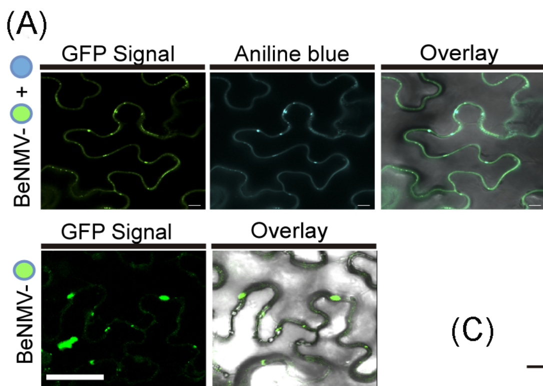
$BeNMV_{295}$

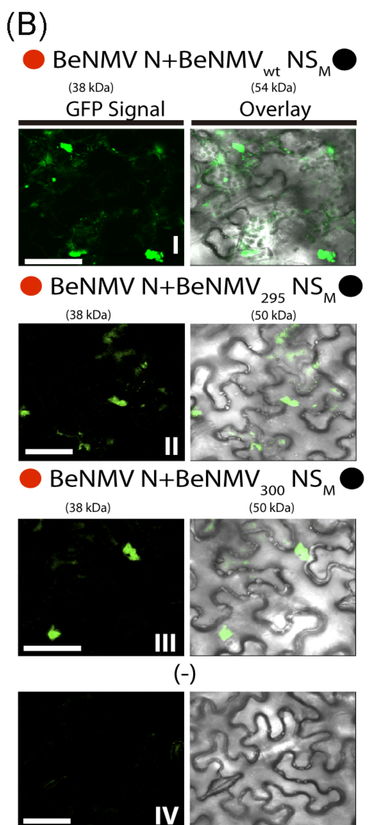
$BeNMV_{300}$

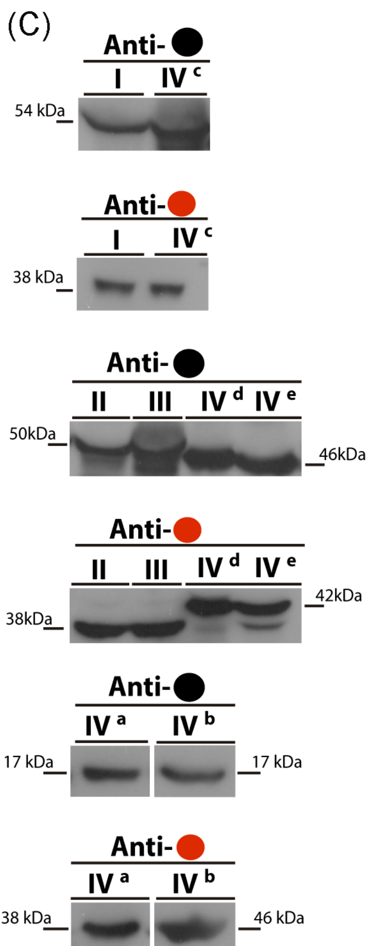
$CSNV_{298}$

$TCSV_{298}$

$TSWV_{298}$


(A)
GFP Signal
Aniline blue
Overlay
BeNMV- +
GFP Signal
Overlay
BeNMV-


(B)
BeNMV N+BeNMV wt NS M
(38 kDa)
(54 kDa)
GFP Signal
Overlay
I
BeNMV N+BeNMV 295 NS M
(38 kDa)
(50 kDa)
II
BeNMV N+BeNMV 300 NS M
(38 kDa)
(50 kDa)
III
(-)
IV
a: N+Nt-YFP ( )
(38 kDa) (17 kDa)
b: NS M + Nt-YFP ( )
(46 kDa) (17 kDa)
c: BeNMV N + BeNMV NS M
(38 kDa) (54 kDa)
d: BeNMV N+BeNMV 295 NS M
(46 kDa) (42 kDa)
e: BeNMV N+BeNMV 300 NS M
(46 kDa) (42 kDa)


(C)
Anti-
I
IV c
54 kDa
Anti-
I
IV c
38 kDa
Anti-
II
III
IV d
IV e
50kDa
46kDa
Anti-
II
III
IV d
IV e
38kDa
42kDa
Anti-
IV a
IV b
17 kDa
17 kDa
Anti-
IV a
IV b
38 kDa
46 kDa