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A self-interacting carmovirus movement protein plays a role in binding of viral RNA during the cell-to-cell movement and shows an actin cytoskeleton dependent location in cell periphery

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ABSTRACT

The p7A of *Melon necrotic spot virus* has been described to be a RNA-binding movement protein essential for cell-to-cell movement but its role in this process is still unknown. Here, we found that primary and secondary structure elements on p7A appear to form a composite RNA-binding site required for both RNA interaction and cell-to-cell movement in plants indicating a direct correlation between these activities. Furthermore, we found that fluorescent-tagged p7A was distributed in punctuate structures at the cell periphery but also in motile cytoplasmic inclusion bodies which were in close association with the actin MFs and most likely generated by self-interacting p7A molecules as shown by BiFC assays. Consistently, the p7A subcellular distribution was revealed to be sensitive to the actin inhibitor, latrunculin B. The involvement of the RNA-binding capabilities and the subcellular location of the p7A in the intracellular and intercellular virus movement is discussed.

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Introduction

Plant viruses move from cell to cell by passing through the plasmodesmata (PD) since, as far it is known, these pathogens are not able to degrade the cell wall. Therefore, the local spread of infection requires not only the movement of viruses from their replication sites to both the plasma membrane and cell wall, but also the modification of the size exclusion limit (SEL) of the PD. To accomplish these functions, viruses encode one or more non-structural factors known as movement proteins (MPs). In spite of the structural differences found among the numerous MPs that have been identified to date, all of them are involved in the transport of infectious material either as entire viral particles or as nucleoprotein complexes. Thus, most MPs share similar properties, such as RNA-binding capacity, interaction with viral and host factors and the PD's gating ability (reviewed in Waigmann et al., 2004 and Lucas, 2006). Moreover, it has been shown that a particular MP is able to complement the movement deficiency of viruses from unrelated families (Scholthof, 2005; Sanchez-Navarro et al., 2006), which indicates a functional equivalence among them. On the other hand, many MPs have been identified as membrane proteins, which are often associated with the endoplasmic reticulum (ER) and cytoskeletal elements, suggesting a great dependence on the cell macromolecular transport system into the viral transport (Nelson and Citovsky, 2005). In this sense, plant viruses have developed a variety of mechanisms to carry out intracellular movement, for example, the *Tobacco mosaic virus* (TMV) MP can be targeted to the PD most likely by using the ER/actin and microtubules network (Boyko et al., 2007; Wright et al., 2007); the multi-component transport system of potex-like and hordei-like viruses (the triple gene block, TGB), which consists in three different proteins (TGBp1, TGBp2 and TGBp3), moves from ER to the cellular periphery generating motile ER-derived bodies (Morozov and Solovyev, 2003), and later, some components (TGBp2 and TGBp3) are recycled by entering into the endocytic pathway (Haupt et al., 2005); the Hsp70 homolog (Hsp70h) of *Beet yellows virus* (BYV) is involved in virion tail assembly and cell-to-cell movement and is autonomously targeted to plasmodesmata in a process mediated by the actomyosin motility system (Prokhnevsky et al., 2005; Avisar et al., 2008). Paradoxically, intracellular and intercellular studies on carmoviruses, which have the simplest multi-component transport system identified to date, are scarce. The cell-to-cell movement of carmoviruses requires the concerted action of two small proteins, no larger than 12 kDa (Cohen et al., 2000; Genoves et al., 2006), which are encoded by a cassette of two genes referred to as the double gene block (DGB) (Hull, 2002). The structural and molecular properties of the corresponding gene products (DGBp1 and DGBp2) have only been studied *in vitro* for both *Carnation mottle virus*, CarMV, and *Melon necrotic spot virus*, MNSV. Biochemical assays have demonstrated that

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homologous DGBp1, characterized as positively charged proteins, are able to bind single-stranded RNA (ssRNA). Moreover, computer predictions combined with circular dicroism data suggested the presence of three different structured domains: an unordered N terminus, an inducible α-helical central region and a C terminus with potential β-sheet folding (Marcos et al., 1999; Akgoz et al., 2001; Vilar et al., 2001, 2005; Navarro et al., 2006). On the other hand, DGBp2 are integral membrane proteins that anchor in the ER through either single- or two-spanning transmembrane domains (TMD) (Vilar et al., 2002; Sauri et al., 2005; Navarro et al., 2006; Martinez-Gil et al., 2007). In this scenario, the ability of carmoviruses to move from cell to cell could be determined by the formation of a hypothetical genome–DGBp1 complex that would be assisted by ER-interacting DGBp2 (Marcos et al., 1999; Vilar et al., 2002). However, speculation concerning this model has only been based on *in vitro* biochemical data.

The MNSV has a single-stranded RNA genome of 4.3 kb which codes for five viral proteins. Both p29 and the read-through p89 are involved in virus replication, and are released from the genomic-length RNA (gRNA), whereas the small p7A and p7B (DGBp1 and DGBp2, respectively), implicated in virus movement, and the coat protein (p42) are translated from the 1.9 and 1.6 kb subgenomic RNAs (sgRNAs), respectively (Riviere and Rochon, 1990; Genoves et al., 2006). We previously described the *in vitro* RNA-binding properties of MNSV p7A (Navarro et al., 2006), and in this research, we have addressed further insight into the p7A contribution on virus cell-to-cell movement. For this purpose, targeted mutations were introduced affecting either the basic amino acids or the α-helix central structure of p7A and the effects of these changes on both the *in vitro* RNA-binding properties and the local spread of MNSV infection were studied by using an infectious vector carrying the green fluorescent protein (GFP). MNSV p7A displayed a strong self-interaction in vitro and in vivo although this property was not required for its RNA-binding capabilities. Furthermore, *in planta* subcellular localization studies of fluorescent–protein fusions combined with colocalization experiments with both viral and cellular markers and bimolecular fluorescence complementation assays showed that the p7A was located in cytoplasmic aggregates or inclusion bodies in close association with the actin cytoskeleton and also in peripheral punctuate structures which may be related with either PD-rich regions or pit fields.

Results

Mutational analyses of the p7A function in MNSV cell-to-cell movement

A large number of virus-encoded MPs bind to single-stranded RNAs in vitro ribonucleoprotein (RNP) complexes to move cell-to-cell throughout the infected plant (Waigmann et al., 2004). RNA-binding activity of p7A has also been reported suggesting that most likely it may be involved in the MNSV genome–MP complex formation (Navarro et al., 2006). In this sense, a study on the involvement of the basic residues and the inducible α-helical secondary structure of the p7A central region in MNSV cell-to-cell movement was performed to map *in vivo* structure-to-function relationships. The experimental approach essentially consisted in the alanine-replacement mutations within the p7A ORF (Fig. 1A) by using the recombinant GFP-MNSV

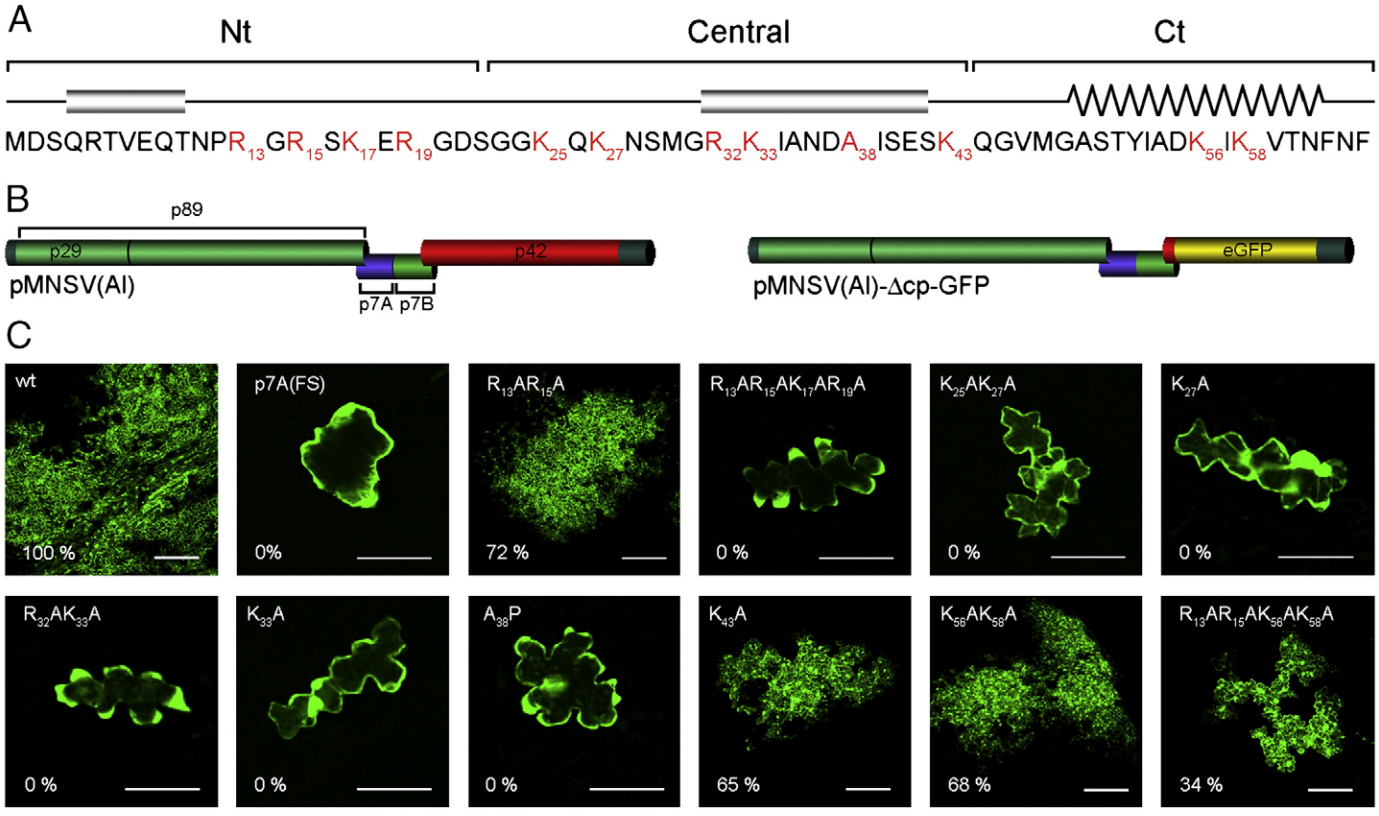

Fig. 1. Site-directed mutagenesis analyses of the p7A function in MNSV cell-to-cell movement in melon plants. (A) Schematic representation of the secondary structure of the p7A showing the three different structural regions. The conserved α-helix and β-sheet structures are represented by boxes and broken lines, respectively. The residues that were modified in p7A ORF are shown in red and their relative position is indicated by a subheading number. (B) A schematic representation of the MNSV genome cloned under the T7 promoter control in the pUC18 vector to generate the pMNSV(AI) infectious vector and the derived MNSV-GFP quimeric chimeric construction (pMNSV(AI)-Δcp-GFP). The names of the viral and fluorescent proteins are indicated. (C) Laser confocal microscopy images taken 2–3 days after the inoculation of each MNSV mutant RNAs on melon cotyledons that were previously agroinfiltrated with the CP-expressing construct pMOG42. The replacements generated in each mutant are indicated. The percentages of movement represented on the right inside the images indicate the infected tissue area generated by each mutant in relation to that produced by the original MNSV RNA. A 0% value indicated the appearance of unicellular foci. Bars in multi- and unicellular foci images correspond to 200 and 20 μm, respectively.

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