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A membrane-associated movement protein of *Pelargonium flower break virus* shows RNA-binding activity and contains a biologically relevant leucine zipper-like motif

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ABSTRACT

Two small viral proteins (DGBp1 and DGBp2) have been proposed to act in a concerted manner to aid intra- and intercellular trafficking of carmoviruses though the distribution of functions and mode of action of each protein partner are not yet clear. Here we have confirmed the requirement of the DGBps of *Pelargonium flower break virus* (PFBV), p7 and p12, for pathogen movement. Studies focused on p12 have shown that it associates to cellular membranes, which is in accordance to its hydrophobic profile and to that reported for several homologs. However, peculiarities that distinguish p12 from other DGBps2 have been found. Firstly, it contains a leucine zipper-like motif which is essential for virus infectivity in plants. Secondly, it has an unusually long and basic N-terminal region that confers RNA binding activity. The results suggest that PFBV p12 may differ mechanistically from related proteins and possible roles of PFBV DGBps are discussed.

Introduction

To establish a productive infection, plant viruses need to move from their replication sites to and through plasmodesmata (PD), which are wall spanning co-axial membranous organelles that bridge the cytoplasm of contiguous cells (Benitez-Alfonso et al., 2010). Such intracellular and intercellular pathogen trafficking can take place in the form of viral particles or nucleoprotein complexes and depends on virus encoded polypeptides named movement proteins (MPs). Though there is considerable structural diversity among MPs of distinct virus taxa (Lucas, 2006), functional properties common to most of them can be outlined including nucleic acid binding capacity, interaction with viral and host factors and ability to increment the size exclusion limit of PD (Nelson and Citovsky, 2005; Waigmann et al., 2004). As viruses seem to hijack the cellular machinery of macromolecular transport to promote their own movement, MPs also often associate with host membrane or cytoskeletal elements (Harries et al., 2010). All these activities can be combined in a single MP or be allocated to several MPs that will assist virus spread in a concerted manner (Hull, 2002; Lucas, 2006).

Pelargonium flower break virus (PFBV) is a member of the genus *Carmovirus* in the family *Tombusviridae*. The monopartite single-stranded (ss) RNA genome of PFBV contains five ORFs that encode polypeptides of 27 (p27), 86 (p86), 7 (p7), 12, (p12) and 37 (p37) kDa (Rico and Hernández, 2004). Reverse genetics experiments have indicated that p27 and its readthrough product p86 (the RNA dependent-RNA polymerase), are essential for viral RNA replication while p37 plays a dual role as capsid protein and as suppressor of RNA silencing (Martínez-Turiño and Hernández, 2009, 2010). According to the available data, cell-to-cell transport of carmo-like viruses in plants is aided by two small virus-encoded polypeptides referred to as the double gene block proteins (DGBps; Hull, 2002). In the case of PFBV, DGBp1 and DGBp2 would correspond to p7 and p12, respectively, on the basis of their similarities with carmoviral proteins with assigned movement function (Genovés et al., 2006; Hacker et al., 1992; Li et al., 1998). Structural and molecular studies performed with *Carnation mottle virus* (CarMV) and *Melon necrotic spot virus* (MNSV) have shown that their DGBps1 (CarMV p7 and MNSV p7A) present RNA binding properties mainly mediated by a basic central region that adopts an α-helical conformation in the presence of secondary structure inducers (Marcos et al., 1999; Navarro et al., 2006; Vilar et al., 2001, 2005). Conversely, DGBps2 of these viruses lack RNA binding activity and contain one (MNSV p7B) or two (CarMV p9) transmembrane domains (TMs) that are targeted and inserted *in vitro* into microsomal membranes derived from the endoplasmic reticulum (ER) (Navarro et al., 2006; Vilar et al., 2002). From these findings, a topological model was advanced in which the cytosolic faced-region (s) of the membrane-associated movement protein (DGBp2) would

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interact with the soluble partner (DGBp1) bound to the viral RNA through their C-terminal regions that might adopt similar β-sheet structures (Navarro et al., 2006; Vilar et al., 2002). The resulting ribonucleotide complex would spread among cells with the aid of an internal membranous translocation system, paralleling that proposed for other MPs (Heinlein and Epel, 2004; Lucas, 2006; Melcher, 2000; Nelson and Citovsky, 2005). As indicated above, this model was mainly based on the properties of DGBps of CarMV and MNSV but *in silico* analyses of the corresponding proteins of other carmoviruses revealed common features that led to the suggestion that the model could apply for most members of the genus (Navarro et al., 2006). Though this was an attractive and straightforward scheme, the mechanism for carmoviral movement is likely more complex and, moreover, virus-specific particularities cannot be discarded. Indeed, most recent results with the DGBps of MNSV have shown that MNSV p7B is targeted *in vivo* to the ER as expected, and delivered to PD via a route that involves the Golgi apparatus (Genovés et al., 2010). However, MNSV p7A is not only able to bind RNA but also to move intracellularly toward PD in the absence of other viral products through a route distinct from that followed by p7B (Genovés et al., 2009). This observation suggests that the distribution of functions among DGBp partners is different or not so well defined as initially proposed.

PFBV p12, like its counterparts in other carmoviruses, contains a high percentage of hydrophobic amino acids distributed along two predicted transmembrane α-helices (Navarro et al., 2006). This trait probably causes the protein to become membrane embedded as described for the homologous CarMV and MNSV proteins and lately for that of *Turnip crinkle virus* (TCV) (Martínez-Gil et al., 2010; Navarro et al., 2006; Vilar et al., 2002). However, several structural peculiarities distinguish PFBV p12 from similar proteins. First, its size is bigger than those of equivalent carmoviral proteins that, with the only exception of the putative DGBp2 of *Japanese iris necrotic ring virus* (Takemoto et al., 2000), range from 7 to 9 kDa (Lommel et al., 2005). Alignment of PFBV p12 with the latter ones indicates that this size gain is primarily due to an N-terminal extension which is rich in basic amino acids (Rico and Hernández, 2004). In addition, p12 contains a seven residue leucine repeat motif, consistent with a leucine zipper, that is absent in related proteins and that is predicted to be four heptads in length (Rico and Hernández, 2004). The natural heterogeneity found among different PFBV isolates argues in favor of the functional relevance of the motif. On one side, only conservative amino acid changes were detected in this region of the molecule and the key leucines of the heptads were strictly preserved despite the large nucleotide variation observed in the corresponding codons (Rico et al., 2006). In addition, an amino acid substitution observed in one isolate would extend the number of heptads of the motif from four to five (Rico et al., 2006).

In order to get insights into the mode of action of PFBV p12, in this work we report experiments aimed at confirming its involvement, together with PFBV p7, in viral movement. We have also investigated the subcellular localization of the protein and the relevance *in vivo* of the putative leucine zipper. Moreover, we have tested whether p12 is endowed with RNA binding activity. Collectively, the results suggest that PFBV p12 may differ mechanistically from related proteins.

Results

Both p7 and p12 are involved in PFBV cell to cell movement

In order to assess whether the two small polypeptides encoded in the central region of PFBV genome, p7 and p12, are involved in viral movement as expected from their homologies with carmoviral proteins of known function (Genovés et al., 2006; Hacker et al., 1992), the start codons of their corresponding ORFs were altered by site-directed mutagenesis using the PFBV infectious clone pSP18F-IC

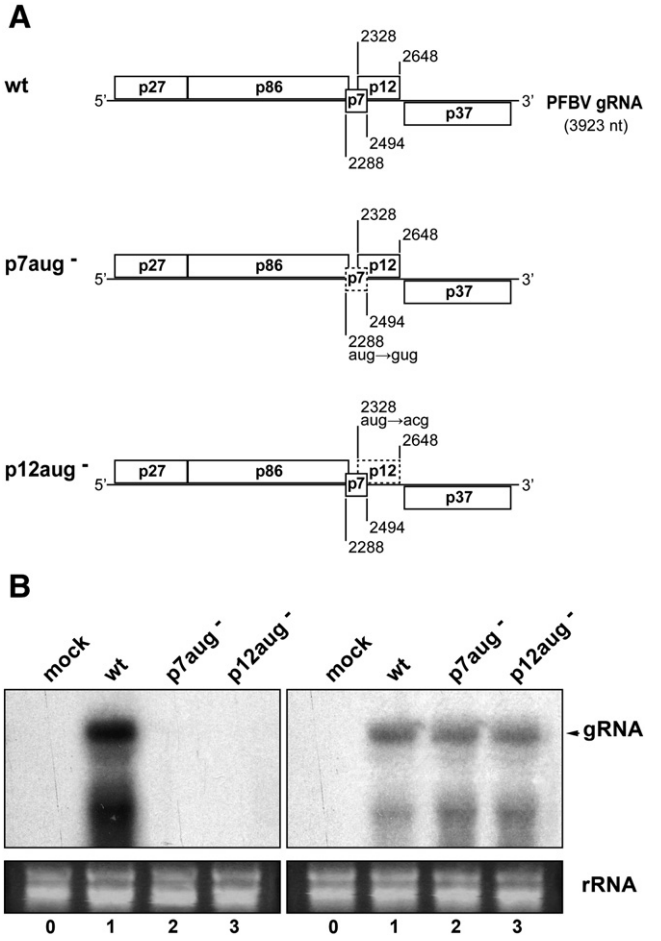

Fig. 1. Bioassay of PFBV mutants. (A) Schematic representation of wt PFBV gRNA (top) and derived mutants p7aug- and p12aug- (bottom). Nucleotide positions of the initiation codons of ORFs p7 and p12 are indicated. Substitutions affecting these codons were introduced into the full-length cDNA clone pSP18-IC leading to triplet changes that are depicted for each mutant. The engineered mutations did not alter the amino acid sequences of overlapping ORFs. In mutants, dashed boxes correspond to ORFs that are not expected to be translated. (B) Northern blot hybridization of total RNA extracted from *C. quinoa* leaves (left panel) or protoplasts (right panel) inoculated with transcripts corresponding to the PFBV wt construct (lane 1) or to mutants p7aug- and p12aug- (lanes 2 and 3, respectively). Lanes 0 correspond to mock-inoculated negative controls. The arrow marks the position of the gRNA; lower bands correspond to subgenomic RNAs. Ethidium bromide staining of rRNA was used as a loading control.

(Rico and Hernández, 2006) as template (Fig. 1A). Run-off transcripts synthesized from the wild type (wt) and the mutated constructs, designated p7aug- and p12aug-, respectively, were used to inoculate *Chenopodium quinoa* leaves and protoplasts. Unlike that observed with the wt construct, no lesions were visible in leaves challenged with the mutant constructs even at 15 days post-inoculation (dpi) and, consistently, no viral-specific signals could be detected by Northern blot hybridization (Fig. 1B). In contrast to that found in plants, mutants p7aug- and p12aug- accumulated in *C. quinoa* protoplasts at levels similar to those of the wt RNA (Fig. 1B). Collectively, the results showed that abolishment of p7 or p12 expression does not affect viral replication but impairs viral spread in leaves supporting the anticipated role of these proteins in cell-to-cell movement.

Amino acid mutations in the leucine zipper-like motif of p12 render movement-defective viruses

To investigate the *in vivo* relevance of the leucine zipper-like motif of p12, nucleotide substitutions leading to single or double replacements of heptadic leucine residues were engineered into the