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

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The movement protein (MP) of *Prunus* necrotic ringspot virus (PNRSV) is required for viral transport. Previous analysis with MPs of other members of the family Bromoviridae has shown that the C-terminal part of these MPs plays a critical role in the interaction with the cognate coat protein (CP) and in cell-to-cell transport. Bimolecular fluorescence complementation and overlay analysis confirm an interaction between the C-terminal 38 aa of PNRSV MP and its cognate CP. Mutational analysis of the C-terminal region of the PNRSV MP revealed that its C-terminal 38 aa are dispensable for virus transport, however, the 4 aa preceding the dispensable C terminus are necessary to target the MP to the plasmodesmata and for the functionality of the protein. The capacity of the PNRSV MP to use either a CP-dependent or a CP-independent cell-to-cell transport is discussed.

Due to the lack of PNRSV infectious clones, a chimera cDNA3 construct between AMV and PNRSV that expresses the green fluorescent protein (GFP), the wild-type (wt) PNRSV MP fused in-frame to the C-terminal 44 aa of AMV MP (A44) and the AMV CP was used (clone pGFP/MP–A44/CP in Sánchez-Navarro et al., 2006; hereafter pMP<sub>pr</sub>–A44). The A44 is required for a specific interaction with the AMV CP to render a functional chimeric AMV/PNRSV RNA 3. The effect of different C-terminal deletions of the PNRSV MP on the functionality of the protein in vivo was examined. MP genes lacking the C-terminal 12, 38 or 42 residues rendered an infection limited to single cells in inoculated plants (Fig. 1). Moreover, the size of the foci was reduced in the plants expressing the AMV P1 and P2 proteins (P12 plants). Deletion of the C-terminal 12 or 38 aa did not block the virus transport. While, deletion of the C-terminal 42 residues rendered an infection limited to single cells (Fig. 1). Moreover, the size of the foci was reduced in the C-terminal 12 and 38 mutants (Supplementary Table S1 available in JGV Online). These results indicate that the C-terminal 38 aa are dispensable for virus transport, but also that residues at position 242–245 are critical for the functionality of the MP. In fact, when position 242–245 were deleted in the wt MP (Fig. 1, pMP<sub>pr</sub>Δ4–A44 construct)
only single infected cells were observed. Previous results obtained with MPs of the 30K family have shown that the dispensable C-terminal portion of the MP plays a role in determining the specificity of the interaction between the MP and CP (Nagano et al., 1997; Sánchez-Navarro et al., 2006). In the case of the chimera AMV constructs this problem was overcome by fusing the A44 at the C terminus of the heterologous MP. To determine if the minimal PNRSV MP C-terminal mutant that is functional in vivo required the presence of A44, we inserted a stop codon after the 245 residue to generate the plasmid pMPP245stop. This construct rendered similar infection foci to that of the pMPP245–A44 chimera (Fig. 1), indicating that the PNRSV MP lacking the C-terminal 38 aa is not inhibited by the presence of the heterologous AMV CP. To analyse the effect of the deleted RNA coding sequences in pMPP245stop–A44 and pMPP241–A44 constructs, the corresponding stop codons were inserted between 245–246 and 241–242 codons in the wt PNRSV MP gene, respectively. Similar results were obtained with these new mutants, indicating that the effects observed are exclusively related to the deleted aa sequences.

To understand why some of the truncated PNRSV MP mutants were defective in virus transport we analysed their subcellular localization. All truncated PNRSV MP genes were fused to the GFP by exchanging the PNRSV MP gene in the binary vector pMPP–GFP (Herranz et al., 2005) to generate pMPP271–GFP, pMPP245–GFP, pMPP241–GFP and pMPPΔ4–GFP plasmids (Fig. 2). The transiently expressed PNRSV MP–GFP fusion protein (MP2–GFP) in Nicotiana benthamiana plants was located in punctate structures at the cell wall as reported previously (Fig. 2a; Herranz et al., 2005). The MP2–GFP co-localized with the well established plasmodesmata marker TMV MP (MP2–DsRed in Fig. 2a). Transient expression of the MP C-terminal mutants supporting virus transport presented the punctuated localization in the cell wall (Fig. 2b, constructs pMPP–GFP, pMPP271–GFP and pMPP245–GFP). However, MP mutants defective in virus transport showed a diffuse fluorescence around the cell wall (Fig. 2b, constructs pMPP241–GFP and pMPPΔ4–GFP), suggesting that these mutants have altered their capacity to interact with the plasmodesmata. In this sense, AMV mutants defective in plasmodesmata localization were also affected in virus transport (Huang et al., 2001). It is worth noting that the C-terminal 32 residues are the most variable part of the PNRSV MP (Aparicio & Pallas, 2002), whereas the region just upstream is highly conserved and contains five sites under negative evolutive selection (V231, D236, R238, T242 and P248), which could be an indication of its importance to maintain the functionality of the protein (Fiore et al., 2008). Our results showed that the small region between residues 242 and 245 (mutant pMPPΔ4–GFP) is critical for both the function of the protein and its subcellular localization. Similar results have been reported.
for the MP of TMV in which the 19 aa preceding the dispensable C-terminal 55 residues are essential for both the localization of the MP to the cell wall and the functionality of the protein (Berna et al., 1991). Thus, this small region could play a role maintaining the three-dimensional structure of the protein or alternatively, represents a potential signal addressed to target the MP to the plasmodesmata. However, we did not find a consensus sequence between the small region of 4 aa and the rest of MPs of the 30K family.

Data obtained with AMV indicate that the virus could traffic through plasmodesmata either as mature virions or by RNA–CP complexes, where the C terminus of AMV MP, although dispensable, could confer specificity to the transport process via an interaction with the cognate CP (Sánchez-Navarro & Bol, 2001; Sánchez-Navarro et al. 2006). Co-variation analysis performed between the MP and CP genes of PNRSV suggested a putative interaction between the C-terminal region of the MP and the N-terminal part of the CP (Codoñer et al., 2006). We analysed the postulated PNRSV MP–CP interaction by in vitro assays and in planta by BiFC. To perform the BiFC assay, the C-terminal fragment of the yellow fluorescent protein (YFP) was fused to the C-terminal part of the full-length and MP mutants, whereas the N-terminal YFP fragment was fused to the PNRSV or AMV CPs (Aparicio et al., 2006). Specific pairs of fusion proteins were transiently co-expressed in N. benthamiana leaves by agroinfiltration. Reconstitution of fluorescence was observed with the combination MPp–CpYFP or MPp271–CpYFP plus NpYFP–CP [Fig. 3a, panels (i) and (ii)]. However, inconclusive results were obtained with the MPp245–CpYFP construct (data not shown). To resolve this problem the possible interaction between MPp245 and the CP was investigated using the overlay assay. The full-length MP and the MPp245 and MPpΔ4 mutants, all carrying the HA epitope at their C termini, were cloned into the protein expression plasmid pDUET (Novagen). The expression of the fusion proteins in Escherichia coli BL21 cells (Novagen) was confirmed by Western blot analysis by using an anti-HA antibody (Fig. 3c). Total protein extracts were electrobotted in duplicate to PVDF membranes and then subjected to overlay assays as described previously (Chen et al., 2000).
The membranes were incubated with either a purified six histidine-tagged PNRSV CP (Fig. 3c, 6hisCP) or a protein extract derived from non-expressing bacteria, as negative control (Fig. 3c, BL21 total). The 6hisCP specifically interacted with the full-length MP and the MP4 mutant, but not with the truncated MP245 (Fig. 3c). No interaction was detected when the membrane was incubated with the negative control. This result confirms the capacity of the PNRSV CP to associate with its cognate MP and suggests that this interaction is mediated exclusively by the C-terminal 38 aa of the MP. To confirm this hypothesis we performed the BiFC assay using only this C-terminal 38 aa (ct38MPp–CYFP). Reconstitution of the fluorescence was observed when ct38MPp–CYFP was co-expressed with NYP–CP [Fig. 3a, panel (iii)]. No signal was detected with the negative control [Fig. 3a, panel (iv)]. Similar negative
results were obtained with the pairs MP$_{271}$–YFP or MP$_{271}$–YFP plus YFP or the AMV CP (not shown). The expression of all fusion proteins used in the BiFC assay was checked by Western blot (Fig. 3b). Our results indicate that the MP–CP interaction is mediated through the C-terminal 38 aa of the MP supporting the previous in silico analysis determining that the PNRSV MP residues interacting with the CP are at positions 253, 256, 257 and 261 (Codoné, et al., 2006). These results, together with previous analysis of MPs assigned to the 30K family, point out an MP scheme in which the majority of the protein (N terminus) is required to allow virus transport. While, the C terminus contains the specific CP interacting determinants. If both regions are independent, MPs lacking the C-terminal region necessary for the CP interaction became non-specific MPs that have the capacity to transport any virus. With this idea we can expect, as well, that different MPs can be functional in a heterologous virus by fusing the specific C-terminal region required for a compatible interaction with the corresponding CP. There are examples for both situations. The MPs of six different viruses are functional in the AMV virus when the A44 is fused at the C terminus of the heterologous MPs (Sánchez-Navarro, et al., 2006). The idea of a non-specific MP is supported by the results presented herein. Thus, the PNRSV MP lacking the C-terminal 38 aa allows the transport of the heterologous AMV. A similar observation has been reported for other bromoviruses: CMV or BMV. In addition, in those viruses it has been reported that the C terminus of the MP could modulate the requirement (CP-dependent) or not (CP-independent) of the CP for virus transport (Akamatsu, Takeda, Kishimoto, et al., 2007; Nagano et al., 2001; Takeda et al., 2005). A similar CP-independent virus transport scenario mediated by the C terminus of the MP could occur in ilarviruses and/or alfamoviruses. However, this aspect cannot be analysed since the CP is also required for other viral functions (e.g. replication and/or translation; Bol, 2005). In the case of the CMV MP, the deletion of the dispensable C terminus region induced an increase of the MP RNA-binding affinity, indicating that perhaps both effects, the lack of the C terminus and the increasing of the RNA affinity, could be required to convert a CP-dependent protein into a CP-independent one (Kim et al., 2004). In BMV, MP–CP interaction requires mature virion formation although an isolate has been described, capable to infect dicot plants, that is able to move between cells independently of CP (Takeda et al., 2004, 2005). In this case, the authors reported that single aa differences at the C terminus of BMV MP are sufficient to alter the requirement for CP in the movement. Apparently, not only the deletion, but also modification of the C terminus could be sufficient to alter the CP requirement of MPs assigned to the 30K family. The open question in this work is to know if the BMV MP variant could be functional in a heterologous virus with an unrelated CP. In the case of the TMV MP, this question has already been answered. The TMV MP has the same scheme of the rest of the MPs in which the C-terminal 55 aa are not necessary for the functionality of the protein. However, and unlike the majority of MPs of the 30K family, the TMV MP functions as a non-specific MP with no other modification of its C terminus (Cooper et al., 1996; Sánchez-Navarro et al., 2006). Regarding the rest of the MPs of the 30K family that have been analysed it can be concluded that the C-terminal region of MPs is associated directly or indirectly with the CP interaction and its deletion or modification is sufficient to generate a non-specific viral transporting protein. In this sense, it will be very interesting to analyse if the deletion of the C-terminal portion responsible for the CP interaction modifies the CP dependence in the group of viruses that are transported as virus particles, e.g. CPMV or CaMV (Carvalho et al., 2003; Stavolone, et al., 2005).

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References


