In vitro and in vivo mapping of the Prunus necrotic ringspot virus coat protein C-terminal dimerization domain by bimolecular fluorescence complementation

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Interactions between viral proteins are critical for virus viability. Bimolecular fluorescent complementation (BiFC) technique determines protein interactions in real-time under almost normal physiological conditions. The coat protein (CP) of Prunus necrotic ringspot virus is required for multiple functions in its replication cycle. In this study, the region involved in CP dimerization has been mapped by BiFC in both bacteria and plant tissue. Full-length and C-terminal deleted forms of the CP gene were fused in-frame to the N- and C-terminal fragments of the yellow fluorescent protein. The BiFC analysis showed that a domain located between residues 9 and 27 from the C-end plays a critical role in dimerization. The importance of this C-terminal region in dimer formation and the applicability of the BiFC technique to analyse viral protein interactions are discussed.

This approach allows the direct real-time visualization of the protein complex under physiological conditions. Until now, BiFC has been mainly used to study interactions among transcription factors from animal, plant and fungal origin in living eukaryotic and bacterial cells (Hu et al., 2002; Walter et al., 2004; Bracha-Drori et al., 2004; Hynes et al., 2004; Wilson et al., 2004; Hoff & Kück, 2005). In the present work, we used BiFC to study the putative CP dimerization capacity of Prunus necrotic ringspot virus (PNRSV) and to map the interaction domain of the PNRSV CP.

PNRSV is a positive-strand RNA plant virus with a tripartite genome that belongs to the genus Ilarivirus. Ilarviruses have the same genome organization, encoding functionally similar translation products, as those of AMV and members of the genera Bromovirus, Cucumovirus and Oleavirus, which belong to the family Bromoviridae. RNAs 1 and 2 encode the replicase subunits P1 and P2, respectively. RNA 3 is translated into the MP, whereas the CP is synthesized from a subgenomic RNA 4. Both MP and CP have RNA-binding properties (Pallás et al., 1999; Aparicio et al., 2003; Herranz & Pallás, 2004; Herranz et al., 2005). Binding of the CP to the 3′ non-translated (3′-NTR) region of viral RNAs is a crucial requirement to establish the infection of AMV and ilarviruses (reviewed by Bol, 2005).

PNRSV and AMV are phylogenetically closely related (Sánchez-Navarro & Pallás, 1997; Codóñer et al., 2004; Codóñer & Elena, 2006). Most studies on the implication of CP in the viral cycle have been conducted in AMV, whereas
there are relatively few experimental data reporting PNRSV CP structural properties and biological functions. PNRSV CP has the capacity to bind to the 3'-NTR of its RNA 4 (Pallás et al., 1999) and can substitute all the AMV CP functions in the replication cycle of a chimeric AMV RNA 3 (Sánchez-Navarro et al., 1997). The RNA-binding domain of PNRSV CP is located at the basic N-terminal region of the CP. We previously demonstrated that CP binding to the 3'-NTR regulates the conformation of the RNA and that the replicase complex of AMV recognizes the 3'-NTR of PNRSV RNA 3, suggesting a similar regulatory mechanism at the 3'-NTR level in AMV and the genera Ilarvirus (Aparicio et al., 2001, 2003). In AMV, CP is involved in the asymmetric plus-strand RNA accumulation, viral RNA translation, virion formation, cell-to-cell movement and the systemic spread of the virus (reviewed by Bol, 2005). In solution, AMV CP occurs as dimers (Kruseman et al., 1971) and CP dimerization is required to obtain wild-type replication level in protoplasts (Choi et al., 2003) to efficiently stimulate RNA translation (Neelamela et al., 2004) and systemic movement (Tenllado & Bol, 2000).

To characterize further the PNRSV CP structural domains, in the present study, we mapped the PNRSV CP region involved in CP–CP interaction by the BiFC approach in both bacteria and intact plant tissues.

To perform BiFC analysis in bacteria, we designed a bacterial-expression strategy that enables the co-expression of bait and prey in the same plasmid. For this purpose, we chose the pETDuet plasmid (Novagen) as the bacterial protein expression vector. This plasmid has two bacteriophage T7 transcription promoters, two multicloning sites (MCS) and a single T7 terminator for the co-expression of two target open reading frames (ORFs). Fig. 1(a) shows a schematic representation of the steps followed to create the fusion constructs. Firstly, fragments corresponding to the N terminus (aa 1–154) and C terminus (aa 153–238) of the EYFP were PCR-amplified and cloned into the MCS to generate pN9Y:CY construct (Fig. 1a). Then, viral ORFs were amplified by PCR and fused to the N9Y and/or CYFP sequences. All fusion proteins contain a 10 aa spacer corresponding to part of the MCS sequence to facilitate the configurational compatibility between bait and prey YFP fusions (Fig. 1b). All constructs were verified by DNA sequencing. Transformed Escherichia coli BL21 (DE3) cells were plated in Luria–Bertani medium with the appropriate antibiotic, and protein expression was achieved with the addition of 1 mM IPTG. Plates were incubated for 48 h at 25°C and reconstitution of YFP fluorescence was observed in single colonies by confocal laser scanning microscopy (CLSM).

As an initial test to validate our system we analysed AMV CP dimer formation. Previous results obtained by the yeast two-hybrid system showed that deletion of the C-terminal 40 aa of the AMV CP abolished dimer formation (Tenllado & Bol, 2000). According to these results, we fused either the full-length or a mutated AMV CP gene lacking the C-terminal 40 aa (Δ40CP), to the split YFP fragments, generating constructs pN9Y-Cpa::CY-Cpa and pN9Y-Cpa::CY-Δ40CP, respectively (Fig. 1b). An additional pN9YSTOP::CYSTOP construct was created to confirm the lack of fluorescence by self-rearrangement of the split YFP fragments (Fig. 1b). No fluorescence signal was observed with the pN9YSTOP::CYSTOP construct, indicating that N9Y and CY fragments themselves did not render self-interaction (Fig. 2a, panel 1). As expected, the pN9Y-Cpa::CY-Cpa construct showed a clear fluorescence signal, whereas the pN9Y-Cpa::CY-Δ40CP plasmid did not (Fig. 2a, panels 2 and 3). In order to confirm that the absence of fluorescent signal was due to the abolition of AMV CP–Δ40CP interaction rather than to an altered protein expression, a Western blot analysis was carried out from total protein extracts. As shown in Fig. 2(b), fusion proteins were clearly detected using an antibody against the CP of AMV (Fig. 2b, panel AMV, lanes 2 and 3). These results demonstrate that this BiFC system can be used to analyse in vitro viral protein–protein interactions.

To study PNRSV CP dimer formation, the full-length CP gene was fused to the two YFP fragments to generate plasmid pN9Y-Cpa::CY-Cpa (Fig. 1b). As can be observed in Fig. 2(a), there was a clear fluorescence signal in bacterial cells transformed with this construct (panel 4), indicating a strong CP–CP interaction. The involvement of the C-terminal arm of CP in dimerization has been reported for several viruses in the family Bromoviridae (Zhao et al., 1995; Choi & Loesch-Fries, 1999; Tenllado & Bol, 2000). Thus, it was reasonable to hypothesize that the C-terminal portion of the PNRSV CP might be the region involved in the CP–CP interaction. A series of CP mutants was generated to analyse the role that this region plays in dimer formation. PNRSV CP genes lacking the C-terminal 9, 18 and 27 aa were exchanged with the corresponding full-length CP gene fused to the CYFP fragment in the plasmid pNY-CPp::CY-CPp. The resultant pNY-CPa::CY-CPa plasmid did not (Fig. 2a, panels 2 and 3). Western blot analysis using an antiserum against the PNRSV CP showed that all mutant CPs were expressed in bacterial cells (Fig. 2b, panel PNRSV, lanes 4, 5, 6 and 7), indicating that a lack of fluorescent signal was unrelated to protein accumulation levels but that it was in fact related to loss of interaction capacity. These results suggest that (i) the C-terminal arm of PNRSV CP plays a critical role in CP–CP interaction and (ii) the domain responsible for the interaction is located between aa residues 9 and 27 from the C-end of the protein. In AMV it has been reported that, in addition to the C-terminal 21 residues, the tryptophan at position 29 (from the C-terminal 40 aa (Δ40CP), to the split YFP fragments, generating constructs pN9Y-Cpa::CY-Cpa and pN9Y-Cpa::CY-Δ40CP, respectively (Fig. 1b). An additional pN9YSTOP::CYSTOP construct was created to confirm the lack of fluorescence by self-rearrangement of the split YFP fragments (Fig. 1b). No fluorescence signal was observed with the pN9YSTOP::CYSTOP construct, indicating that N9Y and CY fragments themselves did not render self-interaction (Fig. 2a, panel 1). As expected, the pN9Y-Cpa::CY-Cpa construct showed a clear fluorescence signal, whereas the pN9Y-Cpa::CY-Δ40CP plasmid did not (Fig. 2a, panels 2 and 3). In order to confirm that the absence of fluorescent signal was due to the abolition of AMV CP–Δ40CP interaction rather than to an altered protein expression, a Western blot analysis was carried out from total protein extracts. As shown in Fig. 2(b), fusion proteins were clearly detected using an antibody against the CP of AMV (Fig. 2b, panel AMV, lanes 2 and 3). These results demonstrate that this BiFC system can be used to analyse in vitro viral protein–protein interactions.

To study PNRSV CP dimer formation, the full-length CP gene was fused to the two YFP fragments to generate plasmid pN9Y-Cpa::CY-CPp (Fig. 1b). As can be observed in Fig. 2(a), there was a clear fluorescence signal in bacterial cells transformed with this construct (panel 4), indicating a strong CP–CP interaction. The involvement of the C-terminal arm of CP in dimerization has been reported for several viruses in the family Bromoviridae (Zhao et al., 1995; Choi & Loesch-Fries, 1999; Tenllado & Bol, 2000). Thus, it was reasonable to hypothesize that the C-terminal portion of the PNRSV CP might be the region involved in the CP–CP interaction. A series of CP mutants was generated to analyse the role that this region plays in dimer formation. PNRSV CP genes lacking the C-terminal 9, 18 and 27 aa were exchanged with the corresponding full-length CP gene fused to the CYFP fragment in the plasmid pNY-CPp::CY-CPp. The resultant pNY-CPa::CY-CPa plasmid did not (Fig. 2a, panels 2 and 3). Western blot analysis using an antiserum against the PNRSV CP showed that all mutant CPs were expressed in bacterial cells (Fig. 2b, panel PNRSV, lanes 4, 5, 6 and 7), indicating that a lack of fluorescent signal was unrelated to protein accumulation levels but that it was in fact related to loss of interaction capacity. These results suggest that (i) the C-terminal arm of PNRSV CP plays a critical role in CP–CP interaction and (ii) the domain responsible for the interaction is located between aa residues 9 and 27 from the C-end of the protein. In AMV it has been reported that, in addition to the C-terminal 21 residues, the tryptophan at position 29 (from the C-terminal 40 aa (Δ40CP), to the split YFP fragments, generating constructs pN9Y-Cpa::CY-Cpa and pN9Y-Cpa::CY-Δ40CP, respectively (Fig. 1b). An additional pN9YSTOP::CYSTOP construct was created to confirm the lack of fluorescence by self-rearrangement of the split YFP fragments (Fig. 1b). No fluorescence signal was observed with the pN9YSTOP::CYSTOP construct, indicating that N9Y and CY fragments themselves did not render self-interaction (Fig. 2a, panel 1). As expected, the pN9Y-Cpa::CY-Cpa construct showed a clear fluorescence signal, whereas the pN9Y-Cpa::CY-Δ40CP plasmid did not (Fig. 2a, panels 2 and 3). In order to confirm that the absence of fluorescent signal was due to the abolition of AMV CP–Δ40CP interaction rather than to an altered protein expression, a Western blot analysis was carried out from total protein extracts. As shown in Fig. 2(b), fusion proteins were clearly detected using an antibody against the CP of AMV (Fig. 2b, panel AMV, lanes 2 and 3). These results demonstrate that this BiFC system can be used to analyse in vitro viral protein–protein interactions.
terminus) is also critical for dimer stabilization by a putative intermolecular interaction mediated via aromatic residues (Choi & Loesch-Fries, 1999). Interestingly, a tyrosine at position 25 of PNRSV CP (from the C terminus; data not shown) is present in a similar position included in the domain described here for CP–CP interaction. In addition, the C-terminal portion of the PNRSV CP sequence is the highest conserved region in the protein among the different isolates sequenced so far (Aparicio et al., 1999), thus supporting the function described here for this region.

Finally, and by considering the close relationship between AMV and PNRSV, we generated the pNY-CPpnrsv::CY-CPamv construct in order to investigate putative heterologous interactions between both CPs. No reconstitution of YFP fluorescence was observed (Fig. 2a, panel 8), although a significant protein expression level of both fusion proteins was detected by Western blot analysis (Fig. 2b, panel AMV+PNRSV, lane 8). Apparently, AMV and PNRSV CPs are unable to interact with each other in spite of the fact that both proteins are interchangeable for different viral processes (Sánchez-Navarro et al., 1997). A plausible explanation for this unexpected observation could rely on the different forms of the virus particles that AMV and PNRSV adopt. Whereas AMV has bacilliform particles, PNRSV forms icosahedral virions. It would be interesting to check if this potential interaction occurs with other true

Fig. 1. Schematic representation of the proteins fused to the two enhanced yellow fluorescent protein (EYFP) fragments. (a) The amino acid sequence of the YFP corresponding to the N-terminal 154 aa (N Y) and C-terminal 84 aa (C Y) were cloned separately into the two MCS of pETDuet plasmid. Next, CP genes of either AMV (CPa) or PNRSV (CPp) were fused to the N Y and C Y sequences, leaving a 10 aa spacer from MCS. This assures sufficient flexibility, and allows for a configurational compatibility between bait and prey YFP fusions. In (b) and (c), constructs were generated to express fusion proteins in E. coli and by transient expression in N. benthamiana leaves, respectively. C-terminal deleted forms of the CP are shown by a triangle (Δ), where numbers correspond to the aa deleted from the end. Crosshatched arrowheads and boxes either represent T7 RNA polymerase or 35S promoter and terminator.
ilarviruses closely related to PNRSV like Apple mosaic virus or Prune dwarf virus.

The BiCF technique offers the possibility of analysing protein interactions in living plant cells (Walter et al., 2004; Bracha-Drori et al., 2004). We decided to analyse the different PNRSV CP–CP interactions in intact plant tissue. For this purpose, the CP fusion proteins were cloned between the cauliflower mosaic virus 35S promoter and the NOS transcriptional terminator in the pMOG800 binary vector (Fig. 1c). Reconstitution of YFP fluorescence was determined by transient co-expression of the desired protein pairs. Nicotiana benthamiana leaves were infiltrated as described previously (Bendahmane et al., 2000) and examined by CLSM at 48 h following infiltration. The results obtained consistently reproduced the pattern generated in bacteria. No fluorescent signal was displayed in leaves that were co-infiltrated with p35S:NY plus p35S:CY cells, indicating the inability of the split YFP fragments to reconstitute YFP fluorescence in vivo (Fig. 3a, panel 1). Leaves co-infiltrated with p35S:NY-CPp plus p35S:CY-CPp and p35S:NY-CPp plus p35S:CY-Δ18CPp rendered a strong YFP fluorescence signal in the cells (Fig. 3a, panels 2 and 3, respectively), whereas a lower intensity signal was detected in leaves co-expressing p35S:NY-CPp and p35S:CY-Δ18CPp (Fig. 3, panel 4). Finally, no YFP reconstitution fluorescence was detected in leaves co-infiltrated with the pair p35S:NY-CPp plus p35S:CY-Δ27CPp (Fig. 3a, panel 5). Analysis of the spectrum properties of the fluorescent signals derived from the PNRSV NY-CP, NY-CPp, NY-CPp and
NY-CP :: CY- Δ18 CP fusion protein interactions showed the typical YFP fluorescence spectrum, which reached a peak around 527 nm (Fig. 3a). Moreover, Western blot analysis against PNRSV CP showed that all fusion proteins accumulated at detectable levels in the infiltrated tissues (Fig. 3b). All these results indicate that YFP fluorescence resulted from protein–protein interactions.

Fig. 3. In vivo BiFC visualization of CP dimerization of PNRSV in N. benthamiana. Three-week-old leaves were co-infiltrated with the indicated pairs of binary vectors: p35S :: NY and p35S :: CY (panel 1), p35S :: NY-CP p and p35S :: CY-CP p (panel 2), p35S :: NY-CP p and p35S :: CY- Δ9 CP p (panel 3), p35S :: NY-CP p and p35S :: CY- Δ18 CP p (panel 4), p35S :: NY-CP p and p35S :: CY- Δ27 CP p (panel 5). Reconstituted YFP fluorescence was monitored with CLSM. Fluorescence emission spectra (II) were measured to confirm the YFP identity emission pattern. (b) Western blot analysis to confirm protein expression of co-infiltrated constructs. Fusion proteins were detected using an antibody against PNRSV CP. Lane numbers correspond to the indicated constructs in (a). The positions of the fusion proteins are indicated. The triangle (Δ) indicates total amino acids deleted from the end.

The present work demonstrates that BiFC technology is an easy and attractive approach to study plant virus protein–protein interactions in intact plant tissue. Finally, the observation that the C-terminal region of the CP of three viruses belonging to three of five genera of the family Bromoviridae (AMV, PNRSV and Cowpea chlorotic mottle virus) is required for dimer formation, tempts us to speculate that this CP behaviour could be a general characteristic of the family.

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