Identification of an N-terminal domain of the plum pox potyvirus CI RNA helicase involved in self-interaction in a yeast two-hybrid system

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Potyvirus CI RNA helicase is a protein involved in RNA genome replication and virus movement. The protein aggregates in the cytoplasm of infected cells to form typical cylindrical inclusions. A yeast two-hybrid system was used to analyse interactions of the CI RNA helicase from plum pox potyvirus (PPV) with itself and with other viral proteins. No interactions could be detected of full-length CI protein with itself or with PPV P3/6K1, NLa, NLb or CP proteins. However, positive self-interactions were detected for N-terminal fragments of the CI protein, allowing the mapping of a CI–CI binding domain to the N-terminal 177 aa of the protein. Further deletion analysis suggested that several regions of this domain contribute to the interaction. Moreover, pull-down experiments demonstrate that, at least in vitro, full-length PPV CI protein is able to self-interact in the absence of other virus or plant factors.

Introduction

Plum pox virus (PPV) is a well-characterized member of the genus *Potyvirus* of plant viruses. Although much knowledge has been gained in the last few years on the molecular biology of potyviruses, very little is known about protein–protein interactions that govern pathogen–host relationships (Revers *et al*., 1999). The genome of the potyviruses consists of a single-stranded messenger-polarity RNA molecule of about 10 kb. It is expressed through translation into a unique polyprotein that undergoes extensive proteolytic processing (Riechmann *et al*., 1992; Shukla *et al*., 1994). One of the unique features of potyvirus infections is the production of characteristic cylindrical inclusions, formed by the virus CI protein, which accumulate in the cytoplasm of the infected cells (Edwarson & Christie, 1996).

The potyvirus CI protein has been shown to have RNA helicase activity (Lain *et al*., 1990; Eagles *et al*., 1994), which is required for virus RNA replication (Fernández *et al*., 1997). Moreover, electron microscopy experiments (Rodríguez-Cerezo *et al*., 1997; Roberts *et al*., 1998) and genetic analysis (Carrington *et al*., 1998) have demonstrated the involvement of CI protein in cell-to-cell movement. Several potyvirus proteins have been suggested to interact with cylindrical inclusions on the basis of immunoreaction of these structures with specific antibodies (Langenberg, 1993; Rodríguez-Cerezo *et al*., 1993; Arbatova *et al*., 1998).

The yeast two-hybrid system (Fields & Song, 1989) was developed to provide a generic approach to identify proteins that interact physically *in vivo* and to define contacts among the subunits of multiprotein complexes, as well as to map specific domains within proteins that are responsible for interactions (Fields & Sternglanz, 1994; Frederickson, 1998). This system relies on the modular nature of many eukaryotic site-specific transcriptional activators to generate a transcriptional signal from the interaction of a protein (X) fused to a DNA-binding domain (DBD) with another protein (Y) fused to a transcription-activation domain (AD). Interaction between proteins X and Y leads to the transcription of a reporter gene containing a binding site for the reconstructed transcriptional activator. The sensitivity and specificity of the two-hybrid system have been enhanced with the introduction of yeast strains such as PJ69/4a (James *et al*., 1996) containing three reporter genes, *HIS3, ADE2* and *lacZ*, each under the control of a different promoter (GAL1, GAL2 and GAL7, respectively), all of which respond to the same activator, GAL4. One of the reporter genes carried by this yeast strain, *GAL2–ADE2*, displays excellent sensitivity and extremely low background, making it ideal for two-hybrid selection.

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In the present study, we report the use of a yeast two-hybrid assay and deletion analysis to map a domain involved in the self-interaction of the PPV CI protein.

Methods

Yeast two-hybrid assay. All media, buffers and methods for the yeast two-hybrid assay were adopted from the MATCHMAKER System 2 protocol (Clontech). Recombinant plasmids were amplified in Escherichia coli strain DH5α and used to transform Saccharomyces cerevisiae PI69/4a (MATa ura3-52 his3-200 trp1-901 leu2-3,112 gal4Δ gal80Δ LYS2:: GAL1–His3 GAL2–ADE2 met2:: GAL7–lacZ) (James et al., 1996).

Sequential or simultaneous yeast transformations were done by using the lithium acetate method of Gietz et al. (1992). Interaction between murine p53,239e (pVA3-1) and SV40 large T antigen,708 (pTD1-1) was used as a positive control (Clontech).

β-Galactosidase activity was tested by colony-lift filter assay (Schneider et al., 1996) or by a quantitative assay in liquid using the Luminescent β-galactosidase detection kit II (Clontech). Accumulation levels of β-galactosidase were estimated on the basis of enzymatic activity of known amounts of purified protein.

Plasmid construction. All plasmids used in this study are listed in Table 1.

Plasmids for full-length protein interactions. PPV CI (nt 3651–5556), P3 (nt 2446–3651) and CP (nt 8578–9566) coding regions were amplified from plasmids pMalCNCIcx (CI) (Fernández et al., 1997) and pPPV (P3/CI and CP) (Riechmann et al., 1990) by PCR with Taq DNA polymerase (Amersham) by using the following oligodeoxynucleotides: P3/CI1, 5’ CATTGCATGGTCTGAAATGGAAGTG 3’ and 5’ CCGGATCCCTACGTGCTGACACGGCTC 3’; CI, 5’ AGCTTGGACGATATAG 3’ and 5’ CTAGCTACGTTAGGG 3’; and CP, 5’ CATTGCATGGTCTGACAAAGAG 3’ and 5’ CGGATCCCTACCTCCCTAC 3’. Restriction sites introduced to facilitate cloning and artificial initiation and termination codons are underlined and in bold, respectively.

The PCR-amplified P3/CI1 fragment was digested with NcoI and BamHI and inserted into pAS2-1 (DBD vector, Clontech) and pACT2 (AD vector, Clontech) to produce pAS-P3/CI and pACT-P3/CI, respectively. The same restriction enzymes were used to insert the CI sequence into pAS2-1 to obtain pAS-CP. pMalCNCIcx contains the PPV CI coding sequence with two point mutations that generate CI178-635 1347–1348 (the last 456 aa of the CI protein) from pAS-CI and pACT-CI, respectively. Deletions in the same restriction enzymes interfere with the enzymatic activities of the protein (Fernández et al., 1997). The CI sequence amplified by PCR from pMalCNCIcx was cloned in the BamHI site of pGAD424 (Clontech) blunt with Klenow enzyme.

The CI sequence from the resulting plasmid was subcloned in pAS2-1 using Cfr91 and SalI sites, producing pAS-CI. pACT-CI was the result of inserting a fragment from pAS-CI obtained by SalI digestion into Smal/NcoI-digested pACT2, filling in with Klenow and further digestion with NcoI. pGAD-Nla was obtained by cloning an NcoI fragment, blunt with Klenow enzyme, from pGGNla (García et al., 1993) that included the PPV Nla sequence into Smal-digested pGAD424. pAS-Nlb, which contains the PPV Nlb coding sequence (nt 7024–8577) cloned in pAS2-1, was supplied by Dr. M. Tsagris (Institute of Molecular Biology & Biotechnology, Heraklion, Greece). To rule out the existence of mutations introduced inadvertently by Taq DNA polymerase, plasmid sequences derived from PCR amplification were either replaced by the corresponding fragments of well-characterized plasmids or sequenced.

Plasmids for deletion mapping. pAS-CI409 and pACT-CI409 were constructed by deleting an ApaI–SalI fragment that includes PPV nt 4878–5555 (the last 226 aa of the CI protein) from pAS-CI and pACT-CI, respectively. Deletion of a PmlI–Sall fragment that includes PPV nt 4182–5355 (the last 438 aa of the CI protein) from pAS-CI and pACT-CI yielded pAS-CI177 and pACT-CI177, respectively. pACT-CI407–635 and pACT-CI407–635 were obtained by deleting an NcoI–ApaI fragment that includes PPV nt 3651–4871 (the first 406 aa of the CI protein) from pAS-CI and pACT-CI, respectively. Deletions in the same plasmids of NcoI–PmlI fragments (including PPV nt 3651–4181, which encode the first 177 aa of CI) yielded pAS-CI178–635 and pACT-CI178–635. pACT-CI134 and pACT-CI134 were constructed by cloning an NcoI–SphI fragment from pACT-CI (PPV nt 3651–4051, encoding the first 134 aa of the CI protein) between the NcoI and SphI sites of pAS2-1 and between the NcoI and SphI sites of pACT-CI, respectively. An

Table 1. Amino acid coordinates of PPV proteins and regions of the PPV polyprotein encoded by plasmids used in the study

<table>
<thead>
<tr>
<th>Protein/plasmid</th>
<th>Coordinates</th>
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<tr>
<td><strong>PPV proteins</strong></td>
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<tr>
<td>P3</td>
<td>767–1116</td>
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<tr>
<td>eK1</td>
<td>1117–1168</td>
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<tr>
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<tr>
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<td>CP</td>
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<td><strong>Binding-domain (DBD) plasmids</strong></td>
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<tr>
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<td><strong>Activation-domain (AD) plasmids</strong></td>
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<td>pGGCI</td>
<td>1169–1803</td>
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Cl self-interaction domain

Nco–Nsi fragment from pACT-CI (PPV nt 3651–3870, encoding the first 73 aa of the CI protein) was cloned between the NcoI and SmaI sites of pAS2-1 and between the NcoI and SacI sites of pACT2, yielding pAS-CI73 and pACT-CI73. pAS-CI76-177 and pACT-CI76-177 were constructed by inserting an NsiI–BamHI fragment from pACT-CI177 (PPV nt 3875–4182, CI aa 76–177) between the SmaI and BamHI sites of pAS2-1 and pACT2, respectively. pACT-CI135-177 was constructed by insertion of an SphI–BamHI fragment from pACT-CI177 (PPV nt 4052–4182, CI aa 135–177) between the NcoI and BamHI sites of pACT2. The same PPV sequence was used to generate pAS-CI135-177 by using an NdeI–BamHI fragment from pACT-CI135-177 in the corresponding sites of pAS2-1.

In all cases, blunting by either Klenow or mung bean nuclease treatment preceded ligation of fragments with non-cohesive ends.

Other plasmids. pMalcNCI was described previously (Fernández et al., 1997). pGGCI was obtained by cloning the PCR-amplified PPV CI coding sequence between the NcoI and SbfI sites of pGG5S6N (Simón-Buela et al., 1997). In pGGCI, the PPV CI coding sequence is under the control of a truncated T7 promoter and the PPV leader sequence (Riechmann et al., 1990). The accuracy of all the constructs was verified by restriction analysis and by sequencing through the junctions of the ligated fragments.

Recovery of plasmids from yeast. Total DNA from yeast was prepared by following the method of Hoffman & Winston (1987) and used to transform E. coli DH5α by electroporation with an Electro manipulator 600 (BTX) according to the manufacturer’s specifications. Colonies were selected in a medium containing ampicillin.

Immunoblot analyses. Yeast cells were grown in appropriate selective synthetic medium SD (Difco) to reach mid-exponential phase (OD₆₀₀ of 0.4–0.6). Total proteins from yeasts were prepared as described in the Yeast Protocols Handbook (Clontech). Yeast cells were lysed with glass beads (425–600 μm) in cracking buffer (8 M urea, 5% SDS, 40 mM Tris–HCl, pH 8.8, 0.1 mM EDTA and 0.4 mg/ml bromophenol blue) supplemented with β-mercaptoethanol and protease inhibitors. Protein samples (between 10 and 20 μg) were fractionated by SDS–PAGE through a 12.5% gel, transferred to nitrocellulose membranes and subjected to Western blot analyses. The membranes were probed with monoclonal antibodies against GAL4 DBD and AD domains (Clontech) and then with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution, Jackson Immunoresearch Laboratories). The peroxidase reaction was developed by using the ECL (Amersham) kit.

In vitro transcription and translation. Translation products were synthesized in a rabbit reticulocyte lysate system (Promega) in the presence of [³⁵S]methionine, as recommended by the manufacturer. The templates were synthetic RNAs (0.2 μg) obtained by in vitro transcription (Amplitube T7 RNA transcription kit, Epicentre) of plasmid pGGCI linearized with PspIvII to obtain full-length CI and with PspIvII and AatII to obtain the CI409 protein fragment. Translation products were analysed by SDS–PAGE as described above, followed by treatment with Amplify (Amersham) and autoradiography of the dried gels at room temperature.

In vitro pull-down assay. E. coli JM109 cells harbouring pMal-c (encoding maltose-binding protein, MBP) and pMalNClI (encoding an MBP–CI fusion protein) were grown, induced and lysed essentially as described previously (Fernández et al., 1995). Appropriate amounts of the crude extract (containing approximately 8 μg of the recombinant proteins) were loaded onto amylose resin columns (New England Biolabs) equilibrated in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 M NaCl. Non-retained proteins were removed by successive washes with the same buffer containing 1, 0.5 and 0.2 M NaCl and no NaCl. Aliquots of 75 μl of the amylose resin with immobilized MBP or MBP–CI proteins were packed in cone tips. Two μl [³²S]labelled in vitro translation products, diluted in 50 μl of 0.2 M NaCl washing buffer, were loaded in each minicolumn and left for 20 min at 4 °C. Minicolumns were washed twice with 200 μl of 10 mM Tris–HCl (pH 7.4) and resin-bound proteins were eluted with the same buffer containing 10 mM maltose. The samples were precipitated with trichloroacetic acid (TCA), resuspended in 20 μl...
Fig. 2. Two-hybrid analysis of interactions among different PPV CI fragments. (A) The genomic organization of PPV. (B) Schematic representation of the CI protein is shown below, with the positions of the seven motifs conserved in RNA helicases. (C) Protein fragments encoded by the pACT-type and pAS-type plasmids used in each experiment are shown in the left columns. The two right columns show the ability to grow on minimal medium containing (+His) or lacking (−His) histidine and adenine and the β-galactosidase activity of yeast cells containing the indicated plasmids. β-Galactosidase assays were carried out in duplicate on transformed yeasts that did not grow in the absence of histidine and adenine, using cells cultured in medium containing these compounds. When the transformed yeasts were able to grow in the presence of histidine and adenine, the mean of β-galactosidase levels (with the standard deviation) of at least eight independent colonies grown in the selective medium is shown. *, Activity at background levels (corresponding to less than 0.5 pg β-galactosidase per µg total protein).
loading buffer (125 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 6 M urea and 5% β-mercaptoethanol), boiled for 5 min and analysed by SDS–12.5% PAGE, Coomassie blue staining and autoradiography.

**Results**

### Interactions of CI protein with itself and with other PPV proteins

We used the yeast strain PJ69/4a in most of the two-hybrid experiments. This strain has three reporter genes, HIS3, ADE2 and lacZ, under the control of promoters that are activated by the GAL4 protein. Although all the reporter genes respond to the presence of this transcriptional activator, they are controlled by different promoter sequences (from the GAL1, GAL2 and GAL7 genes). The use of yeast strains with a variety of inducible promoters confers more reliability on the positive results obtained, since artificial interactions are often not able to activate all of the promoter sequences.

Immuno-electron microscopy experiments have suggested that the CI protein could interact with the proteins P3/6K1 (Rodríguez-Cerezo et al., 1993) and CP (Langenberg, 1993). However, we were not able to detect CI–P3/6K1 interactions by two-hybrid assays using pACT-CI, pAS-CI, pACT-P3/6K1 and pAS-P3/6K1 in both reciprocal crosses. We also analysed CI–NIb, CI–CP and CI–Nla interactions, transforming yeast with pACT-CI and pAS-NIb or pAS-CP, as well as with pAS-Cl and pGAD-Nla, with negative results for all the combinations. In this set of experiments, the only interaction detected was that between NIb and Nla, which has already been reported for two other potyviruses, tobacco vein mottle virus (Hong et al., 1995) and tobacco etch virus (Li et al., 1997).

The fact that CI aggregates in the cytoplasm of infected cells, forming pinwheel inclusions, suggests strongly that it is able to self-interact. Thus, it was surprising that we could not detect the CI–CI interaction in the two-hybrid system with pACT-CI and pAS-CI, regardless of whether the plasmids were introduced in the yeast simultaneously or sequentially. Since enhanced expression of one or both partners might affect the efficiency of interaction in the two-hybrid system either positively or negatively, we also assayed other plasmids that provide different levels of expression of the AD–Cl or DBD–CI fusion products, always with negative results (data not shown). The same negative results were obtained when CI–CI and CI–P3/6K1 interactions were analysed by using fusion proteins with the DBD of LexA or when different yeast strains were used in the experiments (data not shown).

In order to rule out the possibility that the above lack of interaction was the result of instability of either of the plasmids expressing the AD or DBD fusion proteins, single E. coli colonies were analysed after transformation with DNA from yeast doubly transformed with pACT-CI + pAS-CI, pACT-CI + pAS-P3/6K1 or pACT-P3/6K1 + pAS-CI. The presence of both partners was detected for the three pairs of plasmids, although the pACT-type plasmid predominated in all cases (results not shown). In agreement with this result, Western blot analysis of yeast transformed with pACT-CI + pAS-CI demonstrated expression of both CI–AD and CI–DBD fusion proteins but appeared to indicate that CI–AD accumulated at a higher level than CI–DBD (Fig. 1). Nevertheless, a rigorous quantification of the amounts of fusion proteins in yeast cells would require exact measurement of the sensitivity of each monoclonal antibody, a question that we have not addressed.

### Identification of a domain of the CI protein that is able to self-interact

False-negative results in the two-hybrid system have been reported, especially for large proteins in which the interaction domain or the nuclear localization signal could be hidden by the global structure of the fusion protein (Fields & Sternglanz, 1994; Golemis et al., 1997). In order to overcome such possible hindrance, we cloned fragments of the CI sequence in both pAS2-1 and pACT2 vectors in order to obtain the corresponding DBD and AD fusion proteins (Table 1). All CI fusion products were expressed correctly in yeast cells, as confirmed by Western blot analysis (Fig. 1).

No interactions were detected between any of the CI fragments and full-length CI protein (Fig. 2) or any other PPV replication proteins (P3/6K1, Nla, NIb) or CP (data not shown). However, the N-terminal fragments CI177 and CI409 were able to interact with themselves and with each other (Fig. 2). We were not able to detect interactions of the C-terminal fragments CI407-635 and CI178-635 either with themselves or with N-terminal fragments of the CI protein.

Growth of colonies of yeast cells transformed with pACT-CI177 + pAS-CI177 on minimal medium lacking leucine, tryptophan, adenine and histidine could be detected after 3 days incubation at 30°C. However, colonies of yeast cells expressing CI409 + CI409 and CI409 + CI177 were detected in selective medium only after 5 days incubation at 30°C, independently of the vector used to clone the CI fragments. The strengths of the interactions between the different CI fragments were assessed by a liquid β-galactosidase assay (Fig. 2 and data not shown). Yeast cells transformed with pairs of plasmids expressing the N-terminal CI fragments showed levels of β-galactosidase activity 2–4 times higher than those of yeast transformed with the positive-control plasmids pVA3-1 + pTD1-1. Moreover, CI177 self-interaction seemed to confer higher levels of β-galactosidase activity than CI409 self-interaction and CI177–CI409 interactions (Fig. 2). Western blot analysis showed some differences in the levels of accumulation of the different CI fusion proteins, although no correlation could be detected between protein levels and the intensity of the interaction (Fig. 1).

Some extra amino acids derived from vector sequences were linked to the C terminus of the corresponding AD and DBD fusion proteins, as a result of the procedure used to obtain plasmids encoding fragments of the N terminus of the CI protein. We have ruled out the possibility that these extra amino acids are involved in the interactions that we have
detected, since efficient activation of the reporter genes was also observed when plasmids expressing fusion products that ended exactly at PPV CI residue 177 were used in the assay (data not shown).

**Further mapping of the CI interaction domain**

The results described above suggest that sequences involved in self-interaction of the CI protein are confined to its first 177 amino acids. To map the CI–CI interaction domain further, additional interaction experiments were carried out with new fragments from the CI177 N-terminal region cloned in the two-hybrid vectors (Table 1 and Fig. 3). The correct expression of all new fusion proteins was confirmed by Western blot analysis (data not shown). Reporter gene expression was observed in all the combinations that included pAS-CI73 and pAS-CI134. These plasmids were able to activate the transcription of the reporter genes even in combination with empty pACT2 (Fig. 3) or in the absence of a second plasmid encoding the GAL4 AD (data not shown); moreover, yeast cells transformed with these plasmids showed high β-galactosidase activity. This indicates that CI73 and CI134 have intrinsic transcription activation activity and, thus, pAS-CI73 and pAS-CI134 were useless for CI–CI interaction analysis. pACT-CI76-177 by itself did not activate the reporter genes, but together with the empty DBD vector pAS2-1 it was able to confer on yeast the ability to grow in the absence of histidine and adenine, indicating fortuitous interactions between the CI76-177 fragment and pAS2-1 sequences. However, it was possible to differentiate this artifactual reporter gene activation from genuine CI–CI interactions because yeast cells containing plasmids that expressed interacting CI-fusion proteins. No accidental reporter gene activation was observed for the rest of the plasmids, so they provide unambiguous information on the localization of CI–CI binding regions.

Sequences involved in the CI–CI interaction appear to be present at the C-terminal part of the CI177 fragment (aa 135–177), since the CI76-177 and CI135-177 fragments interacted with CI177, irrespective of whether they were fused to AD or DBD, CI76-177 interacted with itself and CI135-177 was able to interact with CI76-177 when the first fragment was fused to DBD and the second to AD (Fig. 3). However, additional sequences upstream of aa 135 are probably
important for the binding, since the CI135-177 fragment was unable to self-interact (Fig. 3). This assumption was supported by the interaction of fragments CI134 and CI177. This 1–134/1–177 interaction appears to involve sequences downstream of aa 73, since the CI73 fragment did not interact with CI177 or any of the other CI fragments assayed (Fig. 3). However, even the first 73 amino acids may play a role in the binding, since AD–CI134 was able to interact with CI177 but not with CI76-177 (Fig. 3). Quantification of \( \beta \)-galactosidase activity by the liquid assay revealed interaction strengths for the smaller fragments comparable to those of the CI177 and CI409 fragments (Fig. 3).

**In vitro self-interaction of the CI protein**

Although the two-hybrid assay revealed protein interactions at the N terminus of the CI protein, it failed to detect self-interaction for the full-length CI protein. To obtain independent evidence of self-interaction of the CI protein at its N-terminal region, we performed pull-down experiments, using CI fused to an MBP tag (MBP–CI) and \( ^{35} \text{S} \)-labelled CI synthesized in an *in vitro* translation system (Fig. 4).

MBP–CI was expressed in *E. coli* and retained on amylose resin. *In vitro*-translated proteins CI and CI409 (N-terminal 409 aa of CI) were incubated at 4°C with an MBP–CI/amylose resin mixture or with an MBP/resin control. After two washing steps, MBP and MBP–CI were eluted from the amylose resin with 10 mM maltose and analysed by SDS–PAGE followed by autoradiography. Both labelled CI and CI409 proteins were able to associate with MBP–CI, but not with MBP alone (Fig. 4).

**Discussion**

The yeast two-hybrid system is a powerful tool for identifying protein–protein interactions in *vivo* and it has already been used to catalogue interactions among a variety of virus gene products (O’Reilly et al., 1997, 1998; Cuconati et al., 1998), including proteins from viruses of the genera *Potyvirus* (Hong et al., 1995; Li et al., 1997; Daro’s et al., 1999; Guo et al., 1999; Merits et al., 1999; Urcuqui-Inchima et al., 1999) and *Tritimovirus* (Choi et al., 2000) of the family *Potyviridae*. In this work, we centred our interest on protein–protein interactions involving the CI protein, which forms cylindrical inclusions...
that accumulate in the cytoplasm of infected cells in all potyvirus infections. Immunoelectron microscopy analysis has suggested that the CI protein could interact with the proteins P1 (Arbatova et al., 1998), P3 (Rodríguez-Cerezo et al., 1993) and CP (Langenberg, 1993). Although we have no data on PPV CI–P1 interactions, both in vitro biochemical assays and in vivo two-hybrid experiments using proteins from potato A potyvirus (PVA) (Merits et al., 1999) and wheat streak mosaic tritimovirus (WSMV) (Choi et al., 2000) confirm that the potyvirus CI and P1 proteins can interact with each other. We have not been able to find interactions between the PPV CI protein and P3/6K1 or CP. Similar results have been obtained with the two-hybrid approach for PVA (Merits et al., 1999) and WSMV (Choi et al. 2000). Interestingly, different in vitro biochemical assays revealed P3–CI interactions for both PVA (Merits et al., 1999) and WSMV (Choi et al., 2000). Thus, the significance of the observed P3–CI interactions is still an open question.

In spite of the fact that CI aggregates to form inclusion bodies in infected cells, we could not detect CI–CI interactions in any of the versions of the two-hybrid system that we have assayed. We cannot discard the possibility that CI requires additional virus or plant factors to interact and form cylindrical inclusions. In this regard, formation of cylindrical inclusions by CI independently expressed in a heterologous system has not been reported. However, we have been able to demonstrate the CI–CI interaction directly by a pull-down experiment in vitro. It is well known that some functional interactions cannot be detected in the two-hybrid system, especially for large proteins, due to protein instability in yeast or to masking in the fusion products of domains involved in protein–protein interaction, nuclear targeting, DNA binding or transcription activation activity. Although we have detected CI–AD and CI–DBD accumulation in doubly transformed yeast, we have no evidence that they reach the nucleus. It is not unusual that interactions that cannot be reproduced in the two-hybrid system using full-length proteins become apparent when protein fragments are assayed. We have observed that a PPV CI N-terminal fragment of 409 aa (CI409) is able to interact with itself but not with the CI fragment. However, CI–CI409 interaction took place in the pull-down assay, which seems to be a more permissive system for CI self-interaction. A smaller PPV CI N-terminal fragment of 177 aa (CI177) was also able to self-interact in two-hybrid assays. Self-interaction of CI177 appeared to be stronger than CI177–CI409 and CI409–CI409 interactions, whereas no CI177–full-length CI interaction was detected, further supporting the view that sequences outside the binding domain obstruct PPV CI–CI interaction in the two-hybrid system. No positive interactions were detected with PPV CI C-terminal fragments. Similar but not identical results have been described for the CI protein of WSMV (Choi et al., 2000). Thus, full-length WSMV CI protein was not able to self-interact in a two-hybrid system, but it did in an in vitro biochemical assay. Moreover, positive interactions were found in two-hybrid assays using fragments of the WSMV CI protein. In contrast with our results, not only a fragment including the N-terminal 209 aa of the protein, but also a 182 aa-long C-terminal fragment showed binding capacity and both were able to interact with full-length CI protein, although no quantitative data on the binding strength were reported. Differences in the PPV and WSMV CI proteins (they show only 29% sequence identity), fragment sizes and experimental conditions could account for the discrepancies between the two results. Overall, we can conclude that a major domain involved in potyvirus CI–CI interaction is positioned at the N-terminal region of the protein, although we cannot rule out the possibility that sequences at the C-terminal region of the protein could also contribute to the binding.

Further deletion analysis of the PPV CI177 fragment did not allow us to delimit a precise self-interaction domain. The results suggest that sequences contributing to the binding are spread across several regions of the CI177 fragment. Moreover, the fact that CI135-177 is able to interact with CI177 but not with itself or with CI76-177 when CI135-177 is fused to AD suggests that interactions could be asymmetric, connecting different sequences of each monomer.

Different types of evidence indicate that the potyvirus CI protein is involved in genome amplification and virus movement (Klein et al., 1994; Fernández et al., 1997; Carrington et al., 1998), but little is known about the involvement of cylindrical inclusions in these functions. Electron microscopy experiments suggest that these structures might be the engines that facilitate virus movement through plasmodesmata (Rodríguez-Cerezo et al, 1997; Roberts et al., 1998), but nothing is known of the role of cylindrical inclusions in the replication function of CI. On the other hand, the NTPase and RNA helicase activities of the potyvirus CI protein have been shown to be essential for virus replication (Fernández et al., 1997). However, extensive mutagenesis analysis has demonstrated that CI mutations that abolish virus replication in isolated cells are not confined to the N-terminal half of the protein, which contains all the motifs conserved in RNA helicases (Carrington et al., 1998). The deleterious effects on virus replication of mutations that affect RNA helicase activity have precluded the assessment of the involvement of this enzymatic activity in the movement function of the CI protein. CI mutations that affect virus movement without appreciable effects on virus replication have also been described. Two mutations that affect cell-to-cell spread and three mutations that allow the formation of multicellular infection loci in inoculated leaves but prevent systemic virus spread map to the N-terminal 125 aa of the tobacco etch potyvirus CI protein, whereas only one or two mutations that affect long-distance virus movement correspond specifically to amino acids from the C-terminal region (Carrington et al., 1998). It is tempting to speculate that the fact that most CI mutations with specific effects on virus movement map to the N-terminal region of the protein, the region that we have shown to be involved in
CI–CI self-interaction, may be related to a requirement of CI for cylindrical inclusion formation in order to perform its role in virus spread. It is clear that further research is required to test this hypothesis and to elucidate the relevance of CI aggregation to the enzymatic activities of the protein and its function in virus replication. The data on CI–CI binding sequences that we describe in this work should help with this task.

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