Self-association and mapping of interaction domains of helper component-proteinase of potato A potyvirus

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Potyviruses possess a single-stranded, positive-sense RNA genome of about 10 kb. The genomic RNA is translated to a large polyprotein which is subsequently processed by three virus-encoded proteases to yield a number of mature proteins (Riechmann et al., 1992). Natural spread of potyviruses is brought about by aphids in a non-persistent manner. Two viral proteins, the helper component-proteinase (HC-Pro) and the coat protein (CP), are required for aphid transmissibility (Pirone & Blanc, 1996). HC-Pro is a multifunctional protein which is also involved in autoproteolytic cleavage of its C terminus from the polyprotein (Carrington et al., 1989), RNA binding (Maia & Bernardi, 1996), genome replication and symptom expression (Atreya & Pirone, 1993; Dolja et al., 1993; Kasschau & Carrington, 1995), virus movement in plants (Cronin et al., 1995; Kasschau et al., 1997; Rojas et al., 1997) and transcription-activation of replication of other viruses (Shi et al., 1997; Pruss et al., 1997). It has been proposed that the HC-Pro of potyviruses could form a homodimer which would be functional in aphid transmission and other biological processes (Thornbury et al., 1985).

The domains of both CP and HC-Pro that are involved in aphid transmission have been characterized to some extent in a few potyviruses. The Asp-Ala-Gly (DAG) motif in the N terminus of potyviral CP is essential for aphid transmissibility (Atreya et al., 1991). At least two conserved motives of HC-Pro, namely the N-terminal KITC and C-proximal PTK, are required for aphid transmission (Atreya & Pirone, 1993; Huet et al., 1994), and the latter may be the binding site for viroins (Peng et al., 1998). The central domain of HC-Pro is associated with genome amplification and long-distance movement (Kasschau et al., 1997).

Isolate B11 of potato A potyvirus (PVA) is non-aphid-transmissible (Rajamäki et al., 1998). Replacement of the B11 CP gene by the corresponding part of the aphid-transmissible isolate U renders isolate B11 aphid-transmissible, indicating that the non-aphid transmissibility of isolate B11 is due to a deficiency in its coat protein while its HC-Pro is functional (Valkonen et al., 1996). This study was carried out to test for the previously proposed interaction between CP and HC-Pro that is involved in aphid transmission (Pirone & Blanc, 1996) and the previously proposed homodimerization of HC-Pro (Thornbury et al., 1985). Data showed that, in PVA, no interaction between HC-Pro and CP was detectable, whereas both HC-Pro and CP showed self-interaction, and are thus probably capable of di- or multimerization.

The yeast two-hybrid system was utilized to detect protein–protein interactions and protein dimerization (Vojtek et al., 1993; Hollenberg et al., 1995). The HC-Pro and CP genes of strain PVA-B11 and the CP gene of strain PVA-U (Puurand et al., 1994; Valkonen et al., 1995) were amplified by PCR and cloned into the two-hybrid vectors pLexA and pVP16 (Hollenberg et al., 1995) or pGAD424 (Clontech). pLexA encodes the DNA-binding protein LexA and contains a selection marker for tryptophan auxotrophy, whereas pVP16 and pGAD424 encode transcription-activation domains and a selection marker for leucine auxotrophy. All the constructs derived from PCR or their deletion mutants were verified by

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**Potyviral helper component-proteinase (HC-Pro)** is a multifunctional protein involved in aphid transmission, long-distance movement, polyprotein processing, genome amplification and symptom expression. It has been proposed that the active form of HC-Pro is a dimer and that coat protein (CP)–HC-Pro interaction is required for aphid transmission. To test these proposed interactions between CP and HC-Pro of potato A potyvirus (PVA), the yeast two-hybrid system was used. HC-Pro was shown to interact with itself in vivo in yeast cells, as did CP. Taken together with previous observations, we conclude that the functional HC-Pro is a homodimer. Deletion analysis showed that a 24 aa domain in the N-terminal half and the C-terminal proteinase part of HC-Pro were required for the interaction between HC-Pro molecules. No interactions were found between HC-Pro and CP using the genes of aphid-transmissible as well as aphid non-transmissible strains of PVA.
Table 1. Interactions between the PVA helper components and coat proteins

<table>
<thead>
<tr>
<th>PVA gene in:</th>
<th>pLexA</th>
<th>pVP16</th>
<th>β-gal filter assay†</th>
<th>β-gal liquid assay‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC^B11</td>
<td>HC^B11</td>
<td>++</td>
<td>37.0 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>CP^B11</td>
<td>CP^B11</td>
<td>+</td>
<td>4.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>HC^B11</td>
<td>CP^B11</td>
<td>−</td>
<td>&lt; 0.5</td>
<td></td>
</tr>
<tr>
<td>CP^B11</td>
<td>HC^B11</td>
<td>−</td>
<td>&lt; 0.5</td>
<td></td>
</tr>
<tr>
<td>CP^U</td>
<td>CP^U</td>
<td>++</td>
<td>13.7 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Empty</td>
<td>Empty</td>
<td>−</td>
<td>&lt; 0.5</td>
<td></td>
</tr>
<tr>
<td>HC^B11</td>
<td>Empty</td>
<td>−</td>
<td>&lt; 0.5</td>
<td></td>
</tr>
<tr>
<td>Empty</td>
<td>CP^B11</td>
<td>−</td>
<td>&lt; 0.5</td>
<td></td>
</tr>
<tr>
<td>Empty</td>
<td>Empty</td>
<td>−</td>
<td>&lt; 0.5</td>
<td></td>
</tr>
</tbody>
</table>

* HC^B11, helper component-protease of PVA strain B11; CP^B11, coat protein of PVA strain B11; CP^U, coat protein of PVA strain U; empty, plasmid without insert. † Qualitative estimate of interaction. ++, colonies turned blue in less than 2 h; +++, colonies turned blue in 2–6 h; +, colonies turned blue in 6–12 h; −, colonies did not turn blue in 12 h. ‡ Units of β-galactosidase activity, calculated using the formula: (A_{420} - 1000)/(A_{660} - 1)v, where A_{420} is the absorbance of the reaction mixture, A_{660} is the cell density of the culture, v is the reaction time in min, and v is the volume (in ml) of culture used for the assay. Values are means ± standard deviation of measurements done with cultures from three independent transformants.

sequencing. DNA manipulations were carried out as described by Sambrook et al. (1989).

The yeast strain L40 [MATa his3Δ200 trp1-901 leu2-3,112 ade2 lys2-801ur3 ura3::(lexAop)16–lacZ] was used for hybrid protein expression. Yeast transformation was performed by the lithium acetate method (Schießl & Gietz, 1989). Transformants were selected by plating on minimal media lacking uracil and tryptophan. To check for expression of fusion proteins, yeast cells were broken by vortexing in Laemmli buffer containing glass beads, and soluble proteins were separated by SDS–PAGE (Laemmli, 1970), blotted onto Immobilon P membrane (Millipore) and probed by LexA monoclonal antibody (Clontech). The blots were developed by the ECL Western blotting detection system (Amersham). The expression of β-galactosidase reporter gene was performed qualitatively by freezing colony filter lifts in liquid nitrogen and subsequently staining with X-Gal in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, pH 7.0, 50 mM β-mercaptoethanol and 0.3 mg/ml X-Gal). The quantitative β-galactosidase activity assays were performed as described by Trawick et al. (1989).

The combinations of fusion plasmids bearing full-length genes of PVA HC-Pro and CP or empty plasmids used for yeast transformations are shown in Table 1. The expression of the fusion proteins was confirmed by Western blot analysis using monoclonal antibody against LexA (Fig. 1a, b). Minor bands below the full-length fusion proteins may represent premature polypeptide or proteolytic degradation products (Fig. 1). Transcription of the reporter gene lacZ can be activated by two interacting hybrid proteins. Thus, the β-galactosidase activity present in transformants can be used as an indicator of interactions between fusion protein partners. In filter lift assays for β-galactosidase activity, transformants carrying the same gene (HC-Pro or CP) in both plasmids had significant β-galactosidase activity as indicated by the appearance of blue colonies. In contrast, the yeast cells transformed with a plasmid combination, in which one plasmid expressed HC-Pro and the other expressed CP of either the aphid-transmissible (PVA-U) or the non-transmissible (PVA-B11) strain, did not show β-galactosidase activity, as indicated by the appearance of white colonies (Table 1). C-terminal fusion of CP in pN-LexA gave the same results as N-terminal fusion in pLexA (data not shown). No β-galactosidase activity was observed in negative controls, in which the cells were transformed with a single fusion plasmid together with the corresponding empty plasmid (Table 1). This indicated that the β-galactosidase activity observed in the combinations of HC-Pro–HC-Pro or CP–CP resulted from a specific interaction of the viral proteins.

The relative strength of HC-Pro and CP self-interactions was quantified by liquid assay of the reporter gene activity. The results obtained were similar to those observed in the filter lift assay (Table 1). The weaker self-interaction of PVA-B11 CP compared to that of PVA-U CP may reflect differences of their expression efficiency or stability in yeast cells (Fig. 1a). The
Homotypic interactions of PVA HC-Pro

Fig. 1. Western blot analysis of expression of PVA CPs (a) and HC-Pro (b) and its mutants (see Fig. 2) in yeast cells. The PVA genes were cloned in the pLexA vector. Total protein prepared from yeast cells (2 A$_{600}$ units) was separated by SDS–PAGE (12%). Monoclonal antibody against LexA was used to detect the fusion proteins. Molecular mass in kDa is indicated on the left. The calculated molecular mass of the LexA fusion part is 26–7 kDa. PVA HC-Pro and CP without fusion are 52 and 30 kDa, respectively.

Fig. 2. Mapping of the domains of PVA HC-Pro involved in self-interaction. Schematic representation of PVA HC-Pro and its mutants. The cDNA of the PVA HC-Pro gene is indicated by the open box and the functions of potyviral HC-Pro domains are shown above. The scale above the box shows the nucleotide position and restriction sites are given below: B, BamHI; X, XbaI; H, HpaI; E, EcoRI; S, SstI; P, PstI. HC-Pro and its mutants are shown as solid lines. The conserved motifs (KITC, PTK and CCC) important for aphid transmission and long-distance movement of potyviruses are given above the lines and the numbers show the amino acid positions. For explanation of ‘+’ and ‘−’ signs, see Table 1 legend. ‘F. P.’ represents a false positive due to activation of reporter gene transcription by one construct alone. (b) Quantitative assay of self-interactions of PVA HC-Pro and its mutants, which were cloned in pLexA and pGAD424. In the HC$_{\Delta BH}$ and HC$_{\Delta PP}$ combination, the former is in pLexA and the latter in pGAD424. CK indicates that two empty plasmids were used. Bars represent average ± standard deviation of β-galactosidase activity (in units, see Table 1) determined in six measurements with three independent colonies.

values of HC-Pro and CP homotypic interactions fall well within the range (3–187 units of β-galactosidase activity) observed for true interacting partners with the same yeast system (Vojtek et al., 1993; Chen et al., 1996). The homotypic interactions of both HC-Pro and CP of PVA thus indicate that they can form homodimers or multimers in yeast cells.

Deletion derivatives of HC-Pro were used to map the regions involved in HC-Pro self-association (Fig. 2 a). Expression of the HC-Pro deletion mutants was confirmed by Western analysis (Fig. 1 b). Deletion of the N-terminal 112 aa (HC$_{\Delta BH}$) reduced reporter gene activity, while deletion of the N-terminal 135 aa (HC$_{\Delta BE}$) and the C-terminal 128 aa (HC$_{\Delta PP}$) completely abolished the interactions between HC-Pro molecules. These results suggest that aa 112–135 and the C-terminal 128 aa are involved in the physical association of HC-Pro. Interactions were also observed between HC-Pro mutants with N-terminal (HC$_{\Delta BH}$) and C-terminal deletions (HC$_{\Delta PP}$) (Fig. 2 b). The HC$_{\Delta BH}$ mutant has a larger deletion but
stronger self-interaction than HCABX, probably due to more favourable folding to expose interaction sites in HCABH (Fig. 2 b). Deletion in the central region of HC-Pro in pLexA (HCASE) resulted in constructs with self-activation of the reporter gene, and the interaction signal for HCASE is therefore regarded as false positive (Fig. 2a).

In this study, HC-Pro was found to interact with itself and two regions in the N- and C-terminal parts of the protein were important for this interaction. These results indicate that homodimerization of HC-Pro may occur. Homodimerization or multimerization of other proteins has also been previously revealed using the yeast two-hybrid system (Chien et al., 1991; Luban et al., 1992). Homodimerization of potyvirus HC-Pro was first proposed based on biochemical studies (Thornbury et al., 1985), and our results strongly support the previous observation. Interactions between N- and C-terminal deletions suggest that the homodimerization of HC-Pro may involve two anti-parallel polypeptide chains.

The PVA CP–CP interaction observed in yeast cells is consistent with the interaction between CP subunits of potyviruses in the process of virus assembly. Potyviral CPs alone can form virus-like, extended helical aggregates (Jagadish et al., 1991). A potyviral CP–CP interaction has previously been reported for tobacco vein mottling potyvirus (TVMV) (Hong et al., 1995).

As noted above, potyviral HC-Pro is a multifunctional protein (reviewed by Maia et al., 1996). The C-terminal one-third of HC-Pro comprises a papain-like proteinase that catalyses autoproteolytic cleavage of its C terminus from the polyprotein (Carrington et al., 1989), and we have shown that this part also plays a crucial role in HC-Pro homodimerization. The N-terminal part of HC-Pro contains a cysteine-rich region with a ‘zinc-finger’-like metal-binding motif, where the KITC motif is embedded. Our deletion analyses indicated that this cysteine-rich region in PVA HC-Pro is not essential for HC-Pro homodimerization whereas a 24 aa stretch of the cysteine-rich region is indispensable. Since SDS–PAGE and Western analysis of PVA HC-Pro fusion protein with or without 2-mercaptoethanol resulted in the same mobility pattern for HC-Pro (data not shown), disulphide bonds may not be involved in HC-Pro homodimerization. The central domain of HC-Pro is important for potyviral long-distance movement (Kasschau et al., 1997), but the role of this region in HC-Pro homodimerization remains unclear due to the deletion construct (HCASE) with self-activation of the reporter gene. The significance of HC-Pro homodimerization with respect to its biological functions remains unresolved.

Unravelling the significance for aphid transmissibility of the relationship between HC-Pro and CP is one of the major research topics in the potyvirus field. The prevailing hypothesis relates that an active HC exhibits two distinct functional domains, one interacting with the viral capsid and the other with the aphid stylet or foregut, thus serving as a bridge between the virus and its vector (Pirone & Blanc, 1996). This hypothesis implies a specific interaction between HC-Pro and CP (or virion). Recently, HC-Pro was shown to interact in vitro specifically with CP monomers or virions originating from aphid-transmissible strains of TVMV (Blanc et al., 1997) and zucchini mosaic potyvirus (ZMV) (Peng et al., 1998), suggesting that direct interaction between potyviral HC-Pro and CP may be involved in aphid transmission. Nevertheless, this proposed interaction of HC-Pro and CP could not be detected for PVA in vivo in yeast cells. Potyvirus transmission by aphids could involve HC-Pro and virion interaction which cannot be assayed in yeast. Our in vitro experiments using a BIACore biosensor and overlay blotting assays have also failed to detect interactions between HC-Pro and PVA virion (data not shown). The failure to detect such interactions for PVA in yeast cells could be due to incorrect post-translational modification and unfavourable folding of the proteins in the yeast nucleus, masking of DNA binding and transcription activation domains or may simply reflect an inherent inability of the two proteins to interact. On the other hand, prefeeding aphids with the N-terminal polypeptide of the CP of maize dwarf mosaic potyvirus expressed in E. coli inhibited aphid transmission of the virus, suggesting direct binding between the CP and the aphid styles (Salomon & Bernardi, 1995). Thus, it is unclear if the interaction between HC-Pro and CP/virion observed for TVMV and ZMV (Blanc et al., 1997; Peng et al., 1998) is a general phenomenon for all potyviruses. Wang et al. (1998) showed that HC-Pro can regulate aphid-transmission specificity, suggesting direct association of HC-Pro with aphid styles. Nevertheless, potyviral HC-Pro itself might not interact directly with CP or virions in the aphid but simply mediate indirectly the binding of virus particles to the aphid by inducing a conformational change of the putative aphid receptor or modifying the chemical environment or charge in aphid mouthparts. Identification of a putative binding site or receptor in the aphid would be helpful to understand the mechanisms of the co-involvement of CP and HC-Pro in aphid transmission.

We are grateful to Mait Mikelsaar and to Leena Ojanen for excellent technical assistance. We thank Professor Jari Valkonen for critical reading of the manuscript and S. M. Hollenberg for providing vectors. The fellowship to D.G. from CIMO and the financial support from Academy of Finland and Biocentrum Helsinki is gratefully acknowledged. M.S. is a fellow of Biocentrum Helsinki.

References


Received 13 November 1998; Accepted 12 January 1999