Effect of mutations within the Cys-rich region of potyvirus helper component-proteinase on self-interaction

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The first ~ 60 amino acids of the N-terminal part of the potyvirus helper component-proteinase (HC-Pro) include highly conserved residues comprising a Cys-rich region. In the present study, the domain in Potato virus Y sufficient for self-interaction was mapped using the yeast two-hybrid system to the 83 N-terminal amino acids of HC-Pro. Mutations in the conserved His and two Cys residues within the Cys-rich region have a strong debilitating effect on self-interaction when introduced in the full-length HC-Pro, but not when introduced in the N-terminal fragment.

The genome of Potato virus Y (PVY), the type member of the genus Potyvirus, contains a monopartite single-stranded RNA of positive polarity. It is translated into a single polyprotein which is processed by three virus-encoded proteinases into the structural and nonstructural proteins necessary for the virus life-cycle (reviewed in Riechmann et al., 1992). Some are these are multifunctional proteins, for example the helper component-proteinase (HC-Pro; reviewed in Maia et al., 1996), the second protein from the N terminus of the polyprotein. This protein has autoproteolytic activity for the release of its own C terminus from the polyprotein, is necessary for systemic movement of the virus within infected plants, is involved in virus amplification and symptom expression, and is required for aphid-transmission of the virus. In addition, the N terminus of HC-Pro from all potyviruses possesses a region containing the highly conserved Lys-Ile-Thr-Cys (KITC) sequence, and it has been shown that the Lys of this sequence is involved in interaction between HC-Pro and the mouthparts of aphids (Blanc et al., 1998). In all potyviruses, this region (which in PVY encompasses amino acids 23 to 56) is rich in Cys residues and contains a highly conserved His residue at position 23 (Fig. 1).

The size of biologically active HC-Pro is between 100 and 150 kDa for PVY and Tobacco vein mottling virus (TVMV; Thornbury et al., 1985). These active forms can be resolved as 58 (PVY) and 53 (TVMV) kDa proteins by denaturing gel electrophoresis, suggesting that HC-Pro is a homodimer. Using the yeast two-hybrid system we have previously shown that the N-terminal half (amino acids 1 to 228) of HC-Pro of isolate PVY-LYE84 is capable of self-interaction (Urcuqui-Inchima et al., 1999). We have now further trimmed HC-Pro, and demonstrate that the 83 N-terminal amino acids are sufficient for self-interaction. Furthermore, we show that the highly conserved C25 and C26 residues within the Cys-rich domain, as well as H23, are involved in HC-Pro self-interaction when present in full-length HC-Pro.

For the two-hybrid experiments, pGADGH-STOP (activation domain) and pLexA (DNA-binding domain; Urcuqui-Inchima et al., 1999) were cleaved by EcoRI/BglII and EcoRI/BglII.

Fig. 1. Schematic representation of full-length wild-type PVY HC-Pro (WT) and the deleted constructs PN1 and PN2. The black lines correspond to the part of HC-Pro deleted in PN1 and PN2. The proteins are represented as rectangles and the amino acid stretch present in each polypeptide is shown to the right. The region encompassing amino acids 23 to 56 is shaded. For the WT and PN2 constructs, the sequence within this region is specified. The amino acids that have been independently mutated are in bold and underlined, with their position provided and the amino acid present in the mutant indicated below.
X-Gal test on filters (not shown) were in line with the quantitative tested in each experiment, and the experiments performed at least three times. The results obtained with the CIBA annealing oligonucleotides 5′ STOP and pLexA, we used an adapter that was obtained by kit. To clone this region (designated PN2) into pGADGH- pT7:HC-Pro, and purified using the QIAquick gel extraction kit (Qiagen). 

Bernardi, 1996) was recovered by BamHI respectively, and purified using the QIAquick gel extraction kit (Qiagen).

The fragment corresponding to the 5′-terminal 249 nucleotides (83 amino acids) of PVY-LYE84 HC-Pro (Maia & Bernardi, 1996) was recovered by EcoRI/Sall digestion of pT7:HC-Pro, and purified using the QIAquick gel extraction kit. To clone this region (designated PN2) into pGADGH-STOP and pLexA, we used an adapter that was obtained by annealing oligonucleotides 5′ TCGACTGTAAAATTAACATCTGTGTAGCTGGT- TTACC3′ and 5′ pGATCTTAATTAATTACAG 3′, carrying the Sall and BglII sites (underlined), and termination codons in all three reading frames. PN1, corresponding to the N-terminal 228 amino acids of PVY HC-Pro, was obtained as described previously (Urcuqui-Inchima et al., 1999).

Four mutants with single amino acid substitutions in the N-terminal Cys-rich region of PVY HC-Pro shown in Fig. 1 were prepared. Point mutants were obtained by site-directed mutagenesis using the Transformer site-directed mutagenesis kit version 2, as specified by Clontech, with the following oligonucleotides (introduced changes underlined):

5′ GATACCCCTCAGATGTTACCTGTGTAAGCTGGTTTACC3′, for mutant H35G
5′ CCCTCAGATCATACGCTGTGTAGCTGGTTTACC3′, for mutant C25G
5′ CTACCCTGAAGTGTTACCTGTGTAAGCTGGTTTACC3′, for mutant K10E
5′ CCCTGTTACAGATGTTACCTGTGTAAGCTGGTTTACC3′, for mutant S35G

The mutants were initially constructed in pMal:NtHC mutants. All recombinant plasmids were sequenced to confirm the presence of the introduced mutations. For quantitative β-galactosidase assays, the transformed yeast colonies were cultured overnight in synthetic selective medium lacking Trp, Leu and His. The overnight cultures were diluted 10-fold with fresh medium and grown to OD500 0.4 to 0.9 before performing the quantitative assays. Cell

<table>
<thead>
<tr>
<th>Table 1. Evaluation of the interactions between PVY HC-Pro and its deleted or mutated constructs</th>
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<tbody>
<tr>
<td>pLexA</td>
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<tr>
<td>HC-Pro</td>
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<tr>
<td>PN1</td>
</tr>
<tr>
<td>PN2</td>
</tr>
<tr>
<td>H35G</td>
</tr>
<tr>
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The mutants were initially constructed in pMal:NtHC mutants. All recombinant plasmids were sequenced to confirm the presence of the introduced mutations. The entire cDNA of wild-type HC-Pro, and HC-Pro with single amino acid substitutions in the full-length protein at H35G, C25C, K10s and S35G contained in pMal:HC-Pro, were released by EcoRI/Xbal and inserted into similarly cleaved pGADGH-STOP. Each of the resulting constructs was cleaved by EcoRI/Xhol and the fragment carrying the HC-Pro gene inserted into pLexA cleaved by EcoRI/Sall. Four PN2 point mutants were prepared by introducing the EcoRI/Sall fragments of the corresponding full-length mutants in either pLexA or pGADGH-STOP carrying the SalI/BglII adapter.

The recombinant plasmids were amplified in Escherichia coli and used to transform Saccharomyces cerevisiae L40 (Le Douarin et al., 1995) by co-transformation as described in the Stratagene HybriZAPr 2.1 kit. Clones were plated on selective medium without Trp, Leu or His (not shown) and their β-galactosidase activities tested on X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). Unrelated sequences [the human proteins Ras and Raf (Vojtek et al., 1993) and murine laminin γ1 (Chang et al., 1996)] were used as positive or negative interaction controls respectively.

For quantitative β-galactosidase assays, the transformed yeast colonies were cultured overnight in synthetic selective medium lacking Trp, Leu and His. The overnight cultures were diluted 10-fold with fresh medium and grown to OD500 0.4 to 0.9 before performing the quantitative assays. Cell
Mutations in HC-Pro self-interaction domain

Table 2. Evaluation of the interactions between PVY PN2 and its mutated constructs

<table>
<thead>
<tr>
<th>pLexA</th>
<th>pGADGH-STOP</th>
<th>β-Galactosidase</th>
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<tbody>
<tr>
<td>PN2</td>
<td>PN2</td>
<td>235 ± 76</td>
</tr>
<tr>
<td>PN2</td>
<td>H²⁵⁶G</td>
<td>243 ± 42</td>
</tr>
<tr>
<td>PN2</td>
<td>C²⁵³G</td>
<td>230 ± 54</td>
</tr>
<tr>
<td>PN2</td>
<td>K²⁹⁶E</td>
<td>388 ± 51</td>
</tr>
<tr>
<td>PN2</td>
<td>C²⁵³G</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>PN²</td>
<td>PN2</td>
<td>210 ± 29</td>
</tr>
<tr>
<td>C²⁵³G</td>
<td>PN2</td>
<td>187 ± 47</td>
</tr>
<tr>
<td>K²⁹⁶E</td>
<td>PN2</td>
<td>272 ± 132</td>
</tr>
<tr>
<td>C²⁵³G</td>
<td>PN2</td>
<td>70 ± 53</td>
</tr>
<tr>
<td>H²⁵⁶G</td>
<td>H²⁵⁶G</td>
<td>206 ± 78</td>
</tr>
<tr>
<td>C²⁵³G</td>
<td>C²⁵³G</td>
<td>120 ± 44</td>
</tr>
<tr>
<td>K²⁹⁶E</td>
<td>K²⁹⁶E</td>
<td>193 ± 23</td>
</tr>
<tr>
<td>C²⁵³G</td>
<td>C²⁵³G</td>
<td>20 ± 20</td>
</tr>
</tbody>
</table>

Permeabilization was performed as described by Guarente (1983). O-Nitrophenyl β-D-galactopyranoside (Sigma) was the chromogenic substrate, and β-galactosidase activity was determined as described by Miller (1972) and expressed as Miller units.

Firstly, the two-hybrid system was used to study possible dimer formation by the N terminus (PN2) of PVY HC-Pro with itself and with wild-type HC-Pro. When tested with itself, PN2 had the same high β-galactosidase activity as did HC-Pro tested with itself (Table 1). Interaction between PN2 and HC-Pro depended on whether PN2 was cloned in pGADGH-STOP or pLexA: higher levels of β-galactosidase activity were observed when PN2 and HC-Pro were cloned in pGADGH-STOP and pLexA respectively than in the converse situation.

PN1 tested with itself produced background levels of β-galactosidase activity, and when tested with wild-type HC-Pro, it yielded higher levels of activity when it was in pGADGH-STOP (Table 1), as already observed (Urcuqui-Inchima et al., 1999). Interaction between PN2 and PN1 led to low β-galactosidase activity, in particular when PN2 was inserted into pGADGH-STOP. The reason why PN1 produces such low levels of activity when tested with itself as opposed to PN2 tested with itself might be due to the presence in PN1, beyond amino acid 83, of sequences inhibiting self-interaction. Removal of these sequences in PN2 would favour interaction.

The N-terminal region of HC-Pro contains the Cys-rich domain. Full-length HC-Pro with the individual point mutations was tested with wild-type HC-Pro (Table 1). The mutation K²⁹⁶E had no detectable effect on self-interaction with wild-type HC-Pro. Mutation of residues H²⁵⁶G, C²⁵³G and C²⁵³G strongly reduced the β-galactosidase activity if the mutated HC-Pro was cloned in pGADGH-STOP and wild-type HC-Pro in pLexA (Table 1). When the mutated HC-Pro were cloned in pLexA and wild-type HC-Pro in pGADGH-STOP, the β-galactosidase activity was 2-fold lower than the activity of wild-type HC-Pro tested with itself.

To test the effect of each mutated residue in HC-Pro on self-interaction, each mutant was tested with itself and with the other mutants. The β-galactosidase activity was close to background levels when each mutant was tested with itself except for K²⁹⁶E, which was as efficient as wild-type HC-Pro with itself (Table 1). With the exception of the K²⁹⁶E mutant, when each mutant was tested with each of the other mutants, activity was dramatically reduced. Interestingly, when K²⁹⁶E was tested with the other mutants contained in pGADGH-STOP or pLexA, β-galactosidase activity was similar to interaction of wild-type HC-Pro tested with each mutant.

PN1 or PN2 tested with each mutated HC-Pro virtually abolished protein–protein interaction (Table 1). Whereas the mutation K²⁹⁶E did not affect self-interaction or interaction with wild-type HC-Pro, the interaction between this mutant and PN1 or PN2 was totally abolished.

The individual point mutants contained in PN2 were also tested against PN2 and each other (Table 2). In all the cases tested, these mutants yielded β-galactosidase activity equivalent or higher than PN2–PN2 interaction, except for mutant C²⁵³G which showed a strongly reduced response.

The results presented here demonstrate that the 83 N-terminal amino acids of PVY HC-Pro (PN2) are sufficient for self-interaction. This is in line with previous results on Lettuce mosaic virus HC-Pro, for which the N-terminal 72 amino acids are sufficient for self-interaction (Urcuqui-Inchima et al., 1999). In an attempt to identify the amino acids in HC-Pro involved in self-interaction, conserved amino acids of the Cys-rich domain were mutated both in full-length HC-Pro and in PN2. It was previously postulated that the Cys-rich domain of PVY HC-Pro could be involved in the formation of a metal-binding site (Robaglia et al., 1989), and moreover well-studied examples of protein dimerization through a zinc finger have appeared (Pan et al., 1989; Chantalat et al., 1999).

In the full-length PVY HC-Pro, the single amino acid changes His²³, Cys²³ and Cys²³ to Gly within the Cys-rich domain are critical for protein–protein interaction (Table 1), whereas this does not seem to be the case for the K²⁹⁶E mutant. In contrast, the results obtained when the same mutations were introduced in PN2 were very different (Table 2): only C²⁵³G showed a marked decrease in β-galactosidase activity as compared to wild-type PN2 or the other mutants. The different behaviour of the mutants whether in full-length HC-Pro or PN2 suggests a more complex situation than anticipated. Various hypotheses can be put forward to explain these discrepancies: (i) the conformation of PN2 whether alone or in the full-length HC-Pro could be different; (ii) the conformation of the mutants could modify the interactions; (iii) the stability of the various constructs could be different; and (iv) other
intra- or inter-molecular interactions could be implicated. As for potyviruses, one cannot exclude the possibility that in different potyviruses different regions of HC-Pro are required for self-interaction. Indeed, other domains have been identified in the case of Potato virus A (Guo et al., 1999), between amino acids 112 and 135 near the N terminus, and between amino acids 329 and 457 at the C terminus.

The precise biological role of the N-terminal region of HC-Pro, in particular with respect to the capacity of this region to self-interact, remains to be established. K²⁰⁰ is an essential residue for transmission and infectivity (Atreya & Pirone, 1993) but does not seem to play a role in self-interaction. On the other hand, H²¹, C²⁵ and C⁵⁵ are essential for the viability of TVMV (Atreya & Pirone, 1993) whereas the entire N-terminal region of Tobacco etch virus is dispensable (Dolja et al., 1993). Our results indicate that these amino acids are dispensable for self-interaction of the N-terminal region whereas they decrease self-interaction of the full-length protein when mutated. These results are in agreement with the suggestions (Atreya & Pirone, 1993) that the N terminus may have important structural features for the proper folding of an active HC-Pro protein.

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References


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