Mutational analysis of plum pox potyvirus polyprotein processing by the NIa protease in *Escherichia coli*

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A binary *Escherichia coli* expression system has been used to study the pathway for proteolytic processing of the plum pox potyvirus (PPV) polyprotein. Trans cleavage at the carboxyl end of the cylindrical inclusion protein occurred, although with lower efficiency than that at the large nuclear inclusion protein–capsid protein junction. No trans cleavage at the carboxyl end of the small nuclear inclusion protein (NIa) was detected. The proteolytic activities at different cleavage sites of several deletion and point mutations of NIa protein have been analysed. The large ΔSX deletion and two different point mutations at His 239 abolished proteolytic activity at all sites. The effect of other mutations, particularly a Glu substitution for Asp 274, depended on the particular cleavage site analysed. The results obtained with the PPV NIa protein mutants were similar to those reported for comparable mutations in the tobacco etch virus 49K protease, despite differences in the sequences recognized for processing. No evident competitive inhibition of the proteolytic activity of PPV NIa protease by the presence of an excess of the different protease mutants could be demonstrated.

**Introduction**

The potyvirus genome consists of a single-component ssRNA of approximately 10000 nucleotides (nt) and is expressed as a large polyprotein precursor which undergoes extensive proteolytic processing to yield mature viral proteins (Carrington & Dougherty, 1987; Hellmann et al., 1988; Carrington et al., 1989; García et al., 1989a). Abundant information has been obtained during recent years on the nature of the proteases and cleavage sites of potyviruses, mainly making use of *in vitro* translation of tobacco etch virus (TEV) RNA prepared by *in vitro* transcription (for a review see Dougherty & Carrington, 1988). The potyviral polyprotein is processed by at least two virus-encoded proteases: the helper component (HC), which is autocatalytically cleaved at its carboxyl end (Carrington et al., 1989), and the small nuclear inclusion protein (49K or NIa protein), which is responsible for the cleavage of the polyprotein at five locations characterized by highly conserved heptapeptide sequences. However, these sequences are recognized with different efficiencies and differ in their ability to be processed in cis and in trans (Carrington et al., 1988; Carrington & Dougherty, 1988; Dougherty et al., 1988, 1989a; Hellmann et al., 1988; Dougherty & Parks, 1989; García et al., 1989b).

The trans proteolytic activity at the large nuclear inclusion protein (NIb)–capsid protein (CP) junction of several plum pox potyvirus (PPV) NIa deletion/substitution mutant proteins has been analysed in an *Escherichia coli* expression system (García et al., 1989a, c). In this paper we have studied the different substrate susceptibilities of several cleavage sites of the PPV polyprotein using these mutants as well as NIa proteases with point mutations at His 239 and Asp 274, which correspond to the His and Asp proposed to form part of the active centre of TEV 49K protease (Dougherty et al., 1989b). Trans activity of the wild-type protease in the presence of an excess of the mutant proteins has also been analysed.

**Methods**

*Bacterial strains and plasmids.* All cloning of plasmids was done in *E. coli* JM109 (Yanisch-Perron et al., 1985). *E. coli* strain C226 (Kunkel et al., 1987) was used in site-directed mutagenesis experiments.

Plasmids pPPV20, pPPV23ΔB5, ΔB7 and ΔB10 have been previously described (García et al., 1989b). pPPV23 and pPPV23ΔSX were obtained by replacing the StuI–SacI (nt 6313 to 9020) or AccI–CclI (nt 5244 to 8309) fragments of pPPV23ΔB7 with the corresponding fragments of pPPV12 and pPPV12ΔSX (García et al., 1989a), respectively. pPPV23H129Q, pPPV23H239L and pPPV23D274E were obtained by site-directed mutagenesis of a HinclI fragment (nt 6284 to 7150) subcloned in a M13 vector, employing the method described by
Kunkel et al. (1987). A mixture of oligodeoxynucleotides pGAATTCGAC(A/T)GTTGTC was used to obtain T→G (H239Q) or AT→TG (H239L) mutations at positions 6420 and 6431 of PPV cDNA, and oligodeoxynucleotide pGGTCGAGAGATAGTG was used to obtain a C→G mutation (D274E) at position 6536.

The numbering of the nucleotide sequence of PPV cDNA and of the amino acid sequence of the PPV Nia protein were taken from Lain et al. (1989) and Garcia et al. (1989a), respectively.

Immunological detection of PPV polypeptides. The preparation of the extracts, separation on SDS-polyacrylamide gels and immunodetection of the proteins transferred to nitrocellulose membranes were as previously described (Garcia et al., 1989b). The anti-PPV CP serum was obtained from rabbits immunized with CP obtained from disrupted virions. Anti-PPV cylindrical inclusion protein (CI) and anti-PPV Nia sera (Martin et al., 1990) were pretreated with a sonicate of E. coli cells to minimize the reaction with bacterial proteins.

Results

Fig. 1 shows a schematic representation of the plasmids encoding the PPV Nia mutant proteases employed in this study. pPPV23ASX carried a frame-conserving deletion in the Nia cistron, resulting in an Nia protein lacking amino acids 201 to 351. The frameshift-inducing deletions of plasmids pPPV23AB5, AB7 and AB10 introduced termination codons near the end of the Nia cistron, which produced proteins in which some amino acids had been removed and others had been added (Garcia et al., 1989a). pPPV23H239Q, pPPV23H239L, and pPPV23D274E contained point mutations at His 239 and Asp 274 which are predicted to be part of the active centre of the Nia protease on the basis of homology to the TEV 49K protease (Dougherty et al., 1989b).

Cleavage at Nib–CP junction

The ability of the mutant proteases to cleave at the Nib–CP junction was investigated by evaluating the amount of CP released from the polyprotein in Western blots of extracts of E. coli cells harbouring high copy number plasmids of the pPPV23 series, encoding either the wild-type or mutant Nia proteases. When indicated, the medium copy number plasmid pPPVS20, which encodes the wild-type Nia protein, was also present. Plasmids of the pPPV23 series encode a truncated PPV CP (32-4K), with the carboxy-terminal 81 amino acids replaced by 48 amino acids encoded by the vector pUC19, whereas pPPVS20 encodes an intact CP (36-6K). In cells
Markers are indicated beside the panels.

Proteolytic cleavage at the CI–6K and NIA–NIB sites

Proteolytic cleavage at the CI–6K, 6K–NIA and NIA–NIB sites would give rise to the accumulation of NIA protein (49.8K) and CI (71.1K) in cells harbouring pPPVS20, or NIA protein and a 50.4K CI-related protein, lacking the first 196 amino acids of CI in cells harbouring plasmids of the pPPV23 series (see Fig. 1). Indeed, CI or truncated CI were the major polypeptides immunoreacting with anti-CI serum in Western blots of cell extracts containing pPPVS20 or pPPV23, which encode wild-type NIA protease (Fig. 3, lanes 7 and 6), indicating that efficient cleavage at the CI–6K junction took place. On the contrary, only minor, or almost undetectable, amounts of NIA protease could be detected in cells containing pPPV23 or pPPVS20, respectively (Fig. 4, lanes 4 and 5). CI and NIA proteins purified from infected leaves or synthesized in E. coli migrated to the same positions in the gel (Fig. 3, lanes 7 and 13; Fig. 4, lanes 4 and 9). As no band with the mobility expected for a 6K–NIA fusion protein was observed, the 6K–NIA junction was probably cleaved. However, because the efficiency of processing at the NIA–NIB site was very poor and free 6K could not be identified, a confident analysis of cleavage at the 6K–NIA site was not possible. Several low mobility bands which immunoreacted with anti-NIA serum were shown in the pPPV23 sample (Fig. 4, lane 4). These probably correspond to polypeptides, initiated at the beginning or internal initiation sites of the ORF, which had been processed at the NIB–CP and CI–6K sites but not at the NIA–NIB junction.

Mutant proteases that did not cleave at the NIB–CP site (ASX, H239Q and H239L) did not do so at the CI–6K site (Fig. 3, lanes 2 to 4); none of these proteases cleaved at the NIA–NIB site (Fig. 4, lane 2 and data not shown). Some truncated CI was detected in D274E extracts (Fig. 3, lane 5), indicating that the CI–6K site was recognized by the D274E mutant, though with much lower efficiency than that of the wild-type protein (see 50.4K :165-2K ratio of lanes 5 and 6, Fig. 3); no free NIA was detected in the D274E sample, indicating that the NIA–NIB junction was not cleaved by this mutant at detectable levels in our system (Fig. 4, lane 3). The bad resolution in the region of low electrophoretic mobility of the gel and the complexity of the pattern, due to the presence of proteins internally initiated, partial processing and, probably, non-specific degradation, precluded a detailed analysis of the high M, polypeptides present in the different extracts. However, the increase of mobility corresponding to the removal of CP by wild-type or D274E protease activity at the NIB–CP site was clearly shown (Fig. 3, lanes 2 to 6; Fig. 4, lanes 2 to 4).

When pPPVS20 was present together with plasmids of the pPPV23 series, besides CI liberated from the polyprotein encoded by pPPVS20 (Fig. 3, lane 7), the CI-related 50.4K protein was also detected in all cases, even when the mutant protease encoded by the pPPV23-type plasmid was inactive (Fig. 3, lanes 8 to 12).
Fig. 3. Immunoblot of cells harbouring plasmids (lanes 1 and 7, pUC19; lanes 2 and 8, pPPV23ASX; lanes 3 and 9, pPPV23H239Q; lanes 4 and 10, pPPV23H239L; lanes 5 and 11, pPPV23D274E; lanes 6 and 12, pPPV23; lanes 14 and 17, pPPV23AB5; lanes 15 and 18, pPPV23AB7; lanes 16 and 19, pPPV23AB10), either alone (lanes 1 to 6) or with pPPVS20 (lanes 7 to 12 and 17 to 19), probed with anti-PPV CI serum. The sample in lane 13 consists of cellular inclusions, containing CI, Nla, Nlb and Nla-Nlb proteins, purified from PPV-infected leaves according to Martin et al. (1990). The arrows indicate the position of CI and of the protein proposed to be encoded by the truncated CI cistron of plasmids of the pPPV23 series. The Mr values of biotinylated protein markers are indicated beside the panels.

It has been previously reported that the mutant protease encoded by pPPV23AB5 was not able to cleave at the Nla–CP site, whereas those encoded by pPPV23AB10 and ΔB7 were partially and almost as active as the wild-type protease, respectively (Garcia et al., 1989a, c). These plasmids differ from the rest of the plasmids described in this paper in that they contain stop codons which interrupt polyprotein synthesis at the end of the Nla cistron (Garcia et al., 1989a) (Fig. 1). They encode a polypeptide of about 104K which could be detected by immunoreaction with anti-CI (Fig. 3, lanes 14 to 16) or anti-Nla (data not shown) sera in extracts of cells harbouring any of the three plasmids. The presence of the 50.4K CI-related protein in the AB7 extracts, and its absence in AB5 and AB10 (Fig. 3, lanes 14 to 16), indicated that AB7, but not AB5 and AB10, protease was able to cleave at the CI–6K site. The absence of a band with the mobility expected for the non-processed truncated CI–6K protein in cells containing pPPV23AB5 and AB10 suggests that the 6K–Nla junction was not recognized by either AB5 or AB10. The wild-type protease encoded by pPPVS20 cleaved the CI–6K site of the polypeptides encoded by pPPV23AB5 and AB10 in trans causing a decrease in the intensity of the 104K bands and the concomitant appearance of the 50-4K CI-related protein (Fig. 3, lanes 17 to 19).

Discussion

In vitro experiments making use of TEV cDNA clones have suggested that the proteolytic maturation of the potyviral polyprotein is a regulated process involving cis and trans cleavages at sites which are recognized with different efficiencies (Carrington et al., 1988; Dougherty & Parks, 1989; Dougherty et al., 1989a). It has been reported that in vitro translated TEV polyprotein is cut by the 49K or Nla protease in trans at the 50K–70K (50K–
CI) and 58K–30K (NIb–CP) sites (although at different rates) and in cis at both sides of the 6K peptide and at the 49K–58K (NIa–NIb) junction (Carrington et al., 1988). NIa-mediated cleavages occurring only in cis have also been reported for tobacco vein mottling virus (Hellmann et al., 1988); trans cleavage at the NIb–CP junction of PPV has been demonstrated in an E. coli expression system (Garcia et al., 1989a). On the other hand, computer-generated modelling and site-directed mutagenesis have shown that the active site of the TEV 49K protease is located in the carboxyl half of the molecule and includes His 234, Asp 269 and Cys 339 (Dougherty et al., 1989b).

Expression of PPV cDNA in E. coli has allowed us to investigate further, by a procedure different from the one used for TEV studies, the proteolytic processing pathway of the potyviral polyprotein. We were also interested in delimiting protease regions involved in catalysis and substrate recognition in order to design inactive proteases which would retain the substrate affinity and be able to compete with the wild-type protease. Point mutations at His 239 and Asp 274 of PPV NIa protease, corresponding to TEV 49K His 234 and Asp 269, have been introduced in a PPV cDNA fragment consisting of 6K₂, NIa, NIb and part of the CI and CP cistrons cloned in pPPV23 (Fig. 1). Different deletion mutations that have been previously described (Garcia et al., 1989a) were also employed in these studies. The results obtained are summarized in Table 1. The 15I amino acid deletion of ΔSX protease rendered it inactive at all cleavage sites analysed, as did the Gln or Leu substitutions for His 239. As a Tyr for His 234 substitution had been previously shown to render the TEV 49K protease inactive for its autocatalytic processing (Dougherty et al., 1989b), our results support the previous identification of this His residue as part of the catalytic triad of the potyviral protease (Dougherty et al., 1989b). Dougherty et al. (1989b) also reported that substituting Glu for Asp at position 269 of the TEV 49K protease resulted in an anomalous proteolytic activity capable of cutting at the 49K N-terminal cleavage site but not at the C-terminal one. Interestingly, the effect of the replacement of the Asp 274 of PPV NIa, which corresponds to Asp 269 of TEV (Garcia et al., 1989a), by Glu also depended on the cleavage site analysed: D274E NIa protease cleaved at the NIb–CP junction almost as efficiently as the wild-type protease and at the CI–6K site with low efficiency, whereas cleavage at the C terminus of the NIa protease was undetectable in our system (Fig. 2, lane 5; Fig. 3, lane 5; Fig. 4, lane 3). Unfortunately, the effects of the D–E substitutions in TEV and PPV proteases could not be directly compared because cleavages at the CI–6K and NIb–CP sites were not analysed in the TEV report (Dougherty et al., 1989b) and the low efficiency of cutting at the NIa–NIb site by the wild-type protease precluded a confident estimation of the effect of the mutation on processing at the borders of the PPV protease. However, we can infer that there are different structural requirements in the protease, probably similar in TEV and PPV, for processing at the various cleavage sites.

Table 1. Proteolytic activity of mutant NIa proteins on different cleavage sites

<table>
<thead>
<tr>
<th>Nla protein</th>
<th>CI–6K</th>
<th>Nla–NIb</th>
<th>NIb–CP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total*</td>
<td>Trans†</td>
<td>Total*</td>
</tr>
<tr>
<td>Wild-type</td>
<td>++ +</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ΔB7</td>
<td>+ +</td>
<td>ND‡</td>
<td>ND</td>
</tr>
<tr>
<td>ΔB10</td>
<td>-</td>
<td>ND‡</td>
<td>ND</td>
</tr>
<tr>
<td>ΔB5</td>
<td>-</td>
<td>ND‡</td>
<td>ND</td>
</tr>
<tr>
<td>D274E</td>
<td>+</td>
<td>ND‡</td>
<td>ND</td>
</tr>
<tr>
<td>H239Q, H239L, ΔSX</td>
<td>-</td>
<td>ND‡</td>
<td>-</td>
</tr>
</tbody>
</table>

* The protease and cleavage site were on the same molecule and both cis and trans cleavages could occur.
† The trans activity of the protease encoded by a medium copy number plasmid was determined on substrates encoded by a high copy number plasmid.
‡ ND, Not determined.
§ Results taken from García et al. (1989a, c).
trans activity at the Nlb–CP junction (Carrington & Dougherty, 1987). PPV AB10 mutant protease, in which the 30 carboxy-terminal amino acids have been replaced by 14 others, showed opposite behaviour; cleavage activity was poor at the Nlb–CP site and undetectable at the CI–6K site and the amino terminus of the protease. Therefore, the carboxyl end of the Nla protease seems to interact with the substrate and the effect caused by different modifications in this region depends on the cleavage site considered.

The results described in this paper are in good agreement with the processing model previously postulated on the basis of in vitro transcription and translation of TEV cDNA clones (Dougherty & Carrington, 1988). The PPV Nlb–CP junction was efficiently processed in trans but the Nla–Nlb site was very poorly cleaved and that seemed to occur only in cis (Fig. 4), as is the case in TEV. The poor processing observed in the E. coli system probably reflects in some way the in vivo situation because large amounts of non-processed Nla–Nlb protein could be isolated from infected leaf extracts (Hiebert et al., 1984; Martin et al., 1990; see Fig. 4, lane 9). Whether non-processed Nla–Nlb protein plays a role in potyvirus replication is an open question. Processing at the CI–6K site also seemed to occur preferentially in cis; however, it is recognized more efficiently than the Nla–Nlb site and trans cleavage by Nla proteases encoded by other plasmids could also take place (Fig. 3, lanes 7 to 12 and 17 to 19).

In a previous report we showed that the activity of PPV Nla protease on the Nlb–CP junction was not affected by the presence of excess of several AB mutant proteases (García et al., 1989c). The results presented in this paper indicate that neither of the two different mutant proteases with Gln or Leu substitutions for the postulated catalytic residue His 239 of the enzyme interfered with cleavage at the Nlb–CP site by wild-type protease (Fig. 2, lanes 8 and 9). Therefore, it is possible to assume that either His 239 is involved in the interaction with the substrate or that the substrate–protease interaction has a rapid turnover, allowing efficient cleavage at the Nlb–CP site by the wild-type protease even in the presence of an excess of mutant protein. The effect of the presence of mutant proteases on cleavage at the CI–6K site by wild-type protein was less clear; Fig. 3 shows that the presence of different plasmids of the pPPV23 series produced variations in the amount of free CI, encoded by pPPV2S20, detected in the extracts (lanes 7 to 12 and 17 to 19). However, because the origin of the numerous bands immunoreacting with anti-CI serum with greater and lesser electrophoretic mobilities than that of CI, which appeared with different intensities in cells harbouring pPPV2S20 together with plasmids of the pPPV23 series (Fig. 3, lanes 7 to 12 and 17 to 19), has not been determined, we cannot judge whether the smaller amount of CI protein was due to degradation of the protein, decrease in its synthesis or inhibition of cleavage at the CI–6K site. In any case, the possibility of inhibiting the Nla protease by mutant proteins is still an open question. Additional data on the nature of the interaction between the protease and different substrates, and on the amino acids involved in the recognition, are required to enable the design of mutant proteins with the ability to interfere with the proteolytic processing of the potyviral polypeptide.

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References


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