Processing of the plum pox virus polyprotein at the P3–6K₁ junction is not required for virus viability

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Proteolytic processing of the potyvirus polyprotein is mainly performed by the virus-encoded NIa protease, whose cleavage sites are characterized by conserved heptapeptide sequences. Partial processing at the cleavage site present between the P3 and 6K₁ cistrons by the plum pox potyvirus (PPV) NIa protease has been previously shown to occur in vitro. We have now studied the role of polyprotein processing at the P3–6K₁ junction in vivo, using a full-length PPV cDNA clone. PPV mutant transcripts containing a histidine for glutamine substitution in the cleavage site sequence (a change that abolishes in vitro processability) are able to infect Nicotiana clevelandii plants, indicating that normal processing at the P3–6K₁ junction is not required for virus viability. However, disease symptoms were not detected and virus accumulation occurred after a second site mutation was introduced into the 6K₁, cistron during replication. This additional change did not restore the in vitro processability of the mutant heptapeptide. Changes at other positions in the heptapeptide (that only slightly altered the in vitro processability of this NIa site) were also engineered and it was found that these mutations affected the time course and severity of the symptom induction process. A possible regulatory effect on the function of the potyvirus P3 + 6K₁ protein by processing at the P3–6K₁ junction is discussed in light of our present results with PPV.

The potyvirus genome, a 10 kb RNA molecule, is expressed through its translation into a unique polyprotein that undergoes extensive proteolytic processing (Riechmann et al., 1992). The virus-encoded NIa protease catalyses most of the proteolytic events, processing the central and C-terminal regions of the polyprotein (Fig. 1). NIa cleavage sites are defined by conserved heptapeptides (amino acids −6 to +1, the scissile bond located between −1 and +1; Dougherty et al., 1988, 1989; Carrington & Dougherty, 1988; García et al., 1989b) and seven of these sequences (named A to F and v; Fig. 1) are found along the potyvirus polyprotein. That these function as NIa cleavage sites has been demonstrated experimentally by N-terminal sequencing of proteins purified from infected tissue (sites B, D, E and F) and by analysis of the products of proteolytic processing (from all seven sites) both in vitro and in Escherichia coli. It is now known that each site has different cleavage peculiarities (cis or trans processing, partial or complete cleavage and different reaction profiles; for review see Riechmann et al., 1992; Dougherty et al., 1990). Nevertheless, there is still controversy over whether the A site is actually used in vivo and, in fact, the nature of the A heptapeptide as an NIa cleavage site has often been overlooked. This heptapeptide is present at similar positions in the polyproteins of all 12 potyviruses sequenced to date (Lain et al., 1989; see below) and it has been shown in cell-free proteolytic processing experiments that the plum pox virus (PPV) site A is partially cleaved in vitro (García et al., 1992). In addition, the presence in plants infected by tobacco vein mottling virus of two proteins of 42 and 37 kDa that immunoreact with antibodies raised against a bacterially expressed 42 kDa protein corresponding to the P3 + 6K₁, cistron (Fig. 1) supports the existence of cleavage site A and suggests that, in vivo, it might also be only partially processed (Rodriguez-Cerezo & Shaw, 1991). Therefore, three different gene products would originate from this region of the potyvirus polyprotein: P3, 6K₁ and P3 + 6K₁. However, in vitro processing at tobacco etch virus (TEV) site A has also been assayed and, in this case, no cleavage was observed (Parks et al., 1992). The presence of the presumed 6K₁ peptide in infected plants has not yet been observed for any potyvirus.

To gain insight into the nature of the A heptapeptide...
as an in vivo NIa cleavage site and to determine whether or not its processing is an essential step in the virus life-cycle, we have introduced several mutations into a full-length PPV cDNA clone (from which infectious transcripts can be synthesized; Riechmann et al., 1990) that alter the PPV A heptapeptide. These mutations had been previously used to determine the partial processing in vitro of PPV site A (Garcia et al., 1992). The Q1116H mutation changes the highly conserved glutamine residue at position -1 (see Fig. 1); it was subsequently shown that this change resulted in a heptapeptide that was unable to be processed in vitro. The Q1111N and S1117A exchanges transform, when introduced together (Q1111N,S1117A mutant), the sequence of the A heptapeptide into that of the F heptapeptide (Fig. 1). The natural NIa cleavage site F (NIb-CP junction) is very efficiently cleaved, both in vitro and in vivo. However, these changes only slightly affected cleavage at the NIa site A.

Fig. 1. Schematic diagram of the mutations introduced into the sequence of the NIa cleavage site A of pGPPV and summary of the results obtained after inoculation of N. clevelandii plants with the corresponding in vitro transcripts. The potyvirus genome is represented in the upper part of the figure; the poly(A) tail is shown as a black rectangle and the VPg as a black circle; the protease responsible for each proteolytic cleavage is indicated. The amino acid sequence at the A and F cleavage sites, as well as the different mutations introduced into the site A sequence, are shown. The presence or absence of symptoms in the inoculated plants is indicated (the number of ‘+’ signs correlating with the severity of the symptoms). The extent of the in vitro processability of the different NIa site A mutant sequences is indicated; ‘+’, partial cleavage; ‘−’, no cleavage. This is a summary of the results presented in Garcia et al. (1992).

These mutations (for details of the construction procedure, see García et al., 1992) were introduced into the full-length PPV clone pGPPV (Riechmann et al, 1990) by fragment replacement, creating plasmids pGPPVQ1116H, pGPPVQ1111N,S1117A, pGPPVQ1111N and pGPPVS1117A (Fig. 1). RNA synthesis from linearized plasmid templates and inoculation of young Nicotiana clevelandii plants with the resulting capped transcripts were performed as previously described (Riechmann et al., 1990, 1991); the transcripts produced in vitro from pGPPV and other cDNA clones were given the prefix ‘t’ (i.e. tPPV). N. clevelandii plants inoculated with tPPV (wild-type) transcripts began to develop typical systemic symptoms in the uninoculated upper leaves 7–10 days after inoculation (Riechmann et al., 1990). All of the 10 plants inoculated with PPVQ1116H mutant transcripts remained asymptomatic during a period of 31 days post-inoculation (d.p.i.). To test whether the Q1116H exchange was a lethal mutation that made tPPVQ1116H unable to replicate (and therefore unable to establish an infection), or was not lethal but hampered in some way the normal virus life-cycle (since no symptoms were detected), crude RNA samples from upper leaves of plants inoculated with tPPVQ1116H were prepared and subjected to RT–PCR as described previously (Riechmann et al., 1991). The oligodeoxynucleotides used as primers for the detection of viral RNA sequences were: primer a, 5' AACAGCTGGGTCGAC 3' (complementary to positions 3627 to 3641 of PPV RNA;
Fig. 2. (a) Detection of viral RNA by RT–PCR in *N. clevelandii* plants inoculated with PPV NIA site A mutant transcripts. PCR amplifications were carried out with crude samples of RNA prepared from uninoculated upper leaves (harvested 15 or 31 d.p.i., as indicated) of plants inoculated with transcripts tPPV (wild-type, lane 3), tPPV Q1116H (lanes 4 and 5), tPPV Q1116H,S117A (lane 6), tPPV Q1116H,S117A (lane 7), tPPV S117A (lane 8) and with an extract of a mock-inoculated plant (lane 2). A control PCR using pGPPV as template is included (lane 1). The region of PPV RNA that was amplified is represented; the positions of the two oligodeoxynucleotides (a and b) used as primers for PCR amplification are shown. (b) Detection of PPV CP in *N. clevelandii* plants inoculated with PPV NIA site A mutant transcripts. Extracts were prepared from uninoculated upper leaves (harvested 15 or 31 d.p.i., as indicated) of plants inoculated with transcripts tPPV (wild-type, lane 2), tPPV Q1116H (lanes 3 and 4), tPPV Q1116H,S117A (lane 5), tPPV Q1116H (lane 6), tPPV Q1116H,S117A (lane 7) and from leaves of a mock-inoculated plant (lane 8). A control sample of purified PPV CP (150 ng) is included (lane 1). The amount of leaf tissue corresponding to the samples is indicated in the lower part of the figure.
mutations was ever detected in the progeny analysis in the absence of the other. These data, together with the identification of the A1123T exchange in the two plants primarily inoculated with tPPVQ1116H that became infected, suggest that this second mutation could be involved in the recovery of the pathogenicity of the virus (although additional mutations in other regions of the PPV genome might have occurred during the infection process). Occasionally, reversion to the wild-type sequence at both positions was observed. These revertants might have already arisen in the originally inoculated plants and then outcompeted the in vivo-generated mutant by selection pressure favourable to wild-type RNA.

To test if the A1123T second-site mutation produced a recovery of the in vitro processability of the Q1116H mutant site A, both changes were introduced together in plasmid pGGP3ΔVwt (Garcia et al., 1992), creating plasmid pGGP3ΔVQ1116H A1123T (Fig. 3b). Translation in a rabbit reticulocyte lysate of transcripts synthesized from these plasmids resulted in a polypeptide with electrophoretic mobility corresponding to its expected size (54-5 kDa; Fig. 3b, lanes 1, 3 and 5). Processing at sites A or B would give rise to N-terminal fragments of...
39.4 and 45.2 kDa, respectively, and polypeptides with these electrophoretic mobilities were readily detected when the translation products derived from tGGP3AV<sup>wt</sup> were incubated with an extract of <i>E. coli</i> cells that express the NIa protease (Fig. 3b, lane 2; proteolytic processing assays were performed as previously described; Garcia <i>et al.</i>, 1992). The histidine for glutamine substitution at position −1 of the A heptapeptide present in products derived from tGGP3AV<sup>Q1116H</sup> precluded the generation of the 39.4 kDa band after the proteolytic incubations (Fig. 3b, lane 4; Garcia <i>et al.</i>, 1992). The processability of this mutant heptapeptide was not rescued by the second mutation A1123T, since the 39.4 kDa band was not generated from products of tGGP3AV<sup>Q1116H,A1123T</sup> either (Fig. 3b, lane 6).

<i>N. clevelandii</i> plants were also inoculated with tPPV<sup>Q1111N,S1117A</sup>, tPPV<sup>Q1111N</sup> and tPPV<sup>S1117A</sup>. While tPPV<sup>Q1111N,S1117A</sup> and tPPV<sup>Q1111N</sup> had a reduced pathogenicity in view of the extremely mild symptoms that they induced, tPPV<sup>S1117A</sup> mutant was more virulent than tPPV<sup>wt</sup> (data not shown). The time course of symptom development was also altered by this mutation; tPPV<sup>S1117A</sup>-inoculated plants developed symptoms 5–7 d.p.i. (i.e., earlier than plants inoculated with wild-type transcripts). Viral RNA was readily detected by RT–PCR at 15 d.p.i. in systemic leaves from plants inoculated with these mutants (Fig. 2a, lanes 6 and 8). Similar high levels of CF were detected in plants inoculated with each of these three mutants and with tPPV<sup>wt</sup>, indicating that the differences in the severity of the induced symptoms apparently did not correlate with differences in virus accumulation (Fig. 2b, compare lanes 2, 5, 6 and 7).

As above, viral RNA sequences were amplified from systemic leaves and the PCR products were sequenced. The presence of Q1111N,S1117A, Q1111N and S1117A mutations in the corresponding viral progeny was confirmed. No <i>in vivo</i>-introduced mutations were detected in the sequenced region. Progeny virus of these three mutants maintained the corresponding parental phenotype with regard to the symptom induction process. All these data indicated that the Q1111N,S1117A, Q1111N and S1117A mutations were stable when in the PPV genome.

As described above, the development of a normal PPV life-cycle was hampered by the Q1116H mutation, yet PPVQ1116H mutant was able to establish an infection in <i>N. clevelandii</i> plants, indicating that a cleavable NIa site A is not an essential requirement for virus viability. This conclusion relies on the assumption that the Q1116H mutation abolishes <i>in vivo</i> processing of the PPV site A in a similar way as it does <i>in vitro</i> (Garcia <i>et al.</i>, 1992), but such assumption is warranted by the current abundance of data on potyvirus proteolytic processing. Changes at the highly conserved −1 position have been engineered in several cleavage sites of different potyviruses and their effects assayed in various systems. They have always been found to abolish the cleavability of the resulting mutant sites (Dougherty <i>et al.</i>, 1988, 1989; García <i>et al.</i>, 1989a). In addition, a replacement of the glutamine residue present at the −1 position of the NIa site E (NIa–NIb junction) (a change similar to Q1116H that also abolishes <i>in vitro</i> cleavability; Garcia <i>et al.</i>, 1989a) was introduced into the PPV genome and the resulting mutant transcripts were unable to establish an infection in <i>N. clevelandii</i> plants (data not shown). Mutation of the −1 glutamine residue of the TEV NIa site D (6K<sub>e</sub>–NIa junction) also eliminates virus viability (Restrepo-Hartwig & Carrington, 1994). These results indicate the capability of −1 glutamine mutations to render viral genomes non-viable when introduced into <i>in vivo</i>-functional NIa sites, and therefore reinforces the interpretation of the processing at NIa site A as somehow dispensable. This dispensability would imply that the full-length product (i.e. P3 + 6K<sub>e</sub>) is the functional protein. On the other hand, the fact that the Q1111N,S1117A, Q1111N and S1117A (as well as other mutations not described in this article; J. L. Riechmann & J. A. García, unpublished results) were viable and stable suggests that a certain degree of tolerance towards amino acid exchanges exists in the heptapeptide and surrounding sequence when a cleavable site is conserved.

<i>In vivo</i> processing of the A site in the normal life-cycle of PPV could be a way to regulate the activity (currently unknown) of the P3 + 6K<sub>e</sub> protein. Such a regulatory role for NIa site A processing seems to be appropriate if the characteristics of this part of the polyprotein are considered, since this site precisely defines the boundary between a region (P3) with a very low level of sequence similarity among different potyviruses and another one (6K<sub>e</sub>) that is much more conserved (Lain <i>et al.</i>, 1989; for additional potyvirus genomic sequences see Johansen <i>et al.</i>, 1991; Bowman Vance <i>et al.</i>, 1992; Jayaram <i>et al.</i>, 1992; Nicolas & Laliberté, 1992; Yeh <i>et al.</i>, 1992; Gough & Shukla, 1993; Puurand <i>et al.</i>, 1994; Gunasinghe <i>et al.</i>, 1994). Being the function of the NIa site A to provide a regulatory step, it is understandable that this site be conserved between a region (P3) with a very low level of sequence similarity among different potyviruses and another one (6K<sub>e</sub>) that is much more conserved (Lain <i>et al.</i>, 1989; for additional potyvirus genomic sequences see Johansen <i>et al.</i>, 1991; Bowman Vance <i>et al.</i>, 1992; Jayaram <i>et al.</i>, 1992; Nicolas & Laliberté, 1992; Yeh <i>et al.</i>, 1992; Gough & Shukla, 1993; Puurand <i>et al.</i>, 1994; Gunasinghe <i>et al.</i>, 1994). Being the function of the NIa site A to provide a regulatory step, it is understandable that this site be present in all the potyviruses sequenced to date. The fact that cleavage at the TEV site A was not detected <i>in vitro</i> (Parks <i>et al.</i>, 1992) could also fit into this model, since TEV may have evolved to make this level of control dispensable. Interestingly, the TEV NIa site A is less well conserved (when compared to the rest of TEV NIa cleavage sites) than those A sites of the rest of potyviruses for which data are available (Riechmann <i>et al.</i>, 1992; Garcia <i>et al.</i>, 1992).

In summary, the fact that tPPVQ1116H was able to initiate an infection indicates that a cleavable NIa site A
is not an essential requirement for such a process. However, the drastic effects that mutations at this cleavage site have on the course of virus infection, as well as the appearance of a second mutation and the reversion to the wild-type sequence, suggest that processing at the P3–6K1 junction plays a relevant role in the life-cycle of PPV and that proper cleavage contributes to the time-course of symptom development and severity.

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