Potato leafroll virus protein P1 contains a serine proteinase domain

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The multi-domain potato leafroll virus replicase protein P1 was expressed in insect cells from the polyhedrin promoter of Autographa californica nucleopolyhedrovirus. Using antisera raised against P1, it was shown that P1 was cleaved near the VPg in insect cells in a manner similar to that in plant cells, to produce a ~27 kDa C-terminal fragment. Furthermore, it was shown that the proposed serine proteinase-like domain within P1 is responsible for this processing and that this can occur in a trans (intermolecular) reaction. Four conserved residues within the serine proteinase domain that are essential for catalysis have been identified, consistent with the proposal that this domain comprises a serine proteinase.

Introduction

Potato leafroll virus (PLRV) is now classified as the type member of the polerovirus genus, within the luteovirus family, which comprises many economically important plant pathogens. The virus genome comprises a monopartite, positive-stranded RNA molecule of ~5.8 kb which is encapsidated in small, isometric particles. Like other luteoviruses, PLRV is obligatorily transmitted by aphids (usually Myzus persicae) in which they are believed not to replicate. In plants, PLRV particles are confined to phloem tissue. Virus particles can be purified in small amounts; they contain a major protein of ~23 kDa, a minor component of 53 kDa and the RNA genome. Isolated viral RNAs are covalently bound to a protein at the 5' terminus (VPg), do not have a significant 3' polyadenylate tail and can be used to establish infections in plant protoplasts (Mayo et al., 1982; Harrison, 1984).

The RNA genomic sequences of a number of luteoviruses have been determined (Mayo et al., 1989; van der Wilk et al., 1989; Keese et al., 1990). The sequence of PLRV revealed eight major ORFs in two blocks of coding sequences (Fig. 1). Full-length cDNA of the luteovirus beet western yellow virus (BYYV), which is closely related to PLRV, was constructed and RNA transcripts were shown to be infectious in protoplasts or by agro-infection in plants (Veidt et al., 1992). Site-directed mutagenetic analyses of BYYV cDNA has established that only the putative polymerase (P1/P2) is required for virus replication, and that only the P3 coat protein is required for virion assembly (Reutenauer et al., 1993). Like many positive ssRNA viruses, PLRV has been found to express the products of its genome in a variety of ways including transcription of subgenomic mRNAs, translational frameshifting, amber stop codon readthrough, internal initiation, and protein processing and maturation. The five 3'-terminal ORFs which characterize the luteovirus group are expressed from two sub-genomic RNAs of ~2.7 kb (Rohde et al., 1994) and ~0.8 kb (Ashoub et al., 1998). The larger subgenomic mRNA encodes the ~23 kDa coat protein P3 (Mayo et al., 1989), the virus transmission protein P5 (Bahner et al., 1990; Brault et al., 1995) and the P4 protein responsible for virus movement between cells (Tacke et al., 1993). The ~0.8 kb sub-genomic mRNA encodes proteins of 71 kDa (P6) and 14 kDa (P7); no function has been assigned to either of these proteins, although P7 has nucleic acid binding properties (Ashoub et al., 1998).

The 5'-coding region specifies proteins of ~28 kDa (P0), ~70 kDa (P1) and a third ORF (P2) translated by frameshift readthrough of the 70 kDa polypeptide to specify a protein of ~118 kDa. No specific function has been assigned to P0, but it appears to be non-essential for replication or transmission (Mayo & Ziegler-Graff, 1996). Starting from the N terminus, P1 has a hydrophobic domain, thought to be involved in membrane attachment, followed by a suggested arrangement of VPg-proteinase-helicase-polymerase (Mayo & Ziegler-Graff, 1996), although others have disputed the presence of helicase motifs (Koonin & Dolja, 1993). The polymerase is thought to be generated by a rare (one in a hundred) translational −1 frameshift from the P1 sequence into the P2 sequence after P1 residue 488 (Prüfer et al., 1992). The suggested P1/P2 protein motif arrangement has led to group
**Fig. 1.** (A) Genome organization of PLRV. The genomic RNA (gRNA) is shown together with the ORFs (boxed areas), numbered according to Ashoub et al. (1998), the arrow indicating the site of the P1–P2 frameshift. The shaded area within P1 indicates the genome position of the PLRV sequences shown in the sequence alignment below. The genomic positions of PLRV sequences encoded in the various plasmid constructs are also indicated. The position of the baculovirus polyhedrin promoter is shown by arrowheads. (B) Alignment of luteovirus P1 amino acid sequences comprising the putative proteinase domain (PLRVsco, potato leafroll virus, Scottish isolate; BMYV, beet mild yellowing virus; CAYV, cucurbit aphid-borne yellows virus; BIFI, beet mild yellowing virus, French isolate; BYDVP, beet mild yellowing virus, Chinese isolate; PEM, potato leafroll virus, Dutch isolate).
II luteoviruses, including PLRV, being classified into the picornavirus-like supergroup of viruses (Miller et al., 1995; Mayo & Ziegler-Graff, 1996). The PLRV P1 sequence has, like all of the former subgroup II luteoviruses, a serine proteinase motif H(x-25)D/E(x70–80)TR/KxGxSG (Habili & Symons, 1989; Gorbaleynia et al., 1989; Miller et al., 1995; Mayo & Ziegler-Graff, 1996). van der Wilk et al. (1997) demonstrated, however, that the VPg was closely downstream, rather than upstream, of the proposed serine proteinase domain, with related sequences also being present in other group II luteoviruses. This arrangement was unexpected since in all other ssRNA viruses with a VPg, the arrangement VPg-proteinase-polymerase is found.

Recently, Früher et al. (1999) showed that P1 is processed in plants infected with PLRV. A major product of this processing is a 25 kDa protein representing the C terminus of P1 (P1-C25); the N terminus of the P1-C25 is either the VPg domain, or closely adjacent to it. It was strongly suspected that this cleavage was due to the closely adjacent serine proteinase motif. Supporting this hypothesis, experiments in which conserved residues within the serine proteinase active site motif were mutagenized showed that RNAs of BWYV which were replication-competent were no longer so. Additionally, the P1 of subgroup I luteoviruses, which is significantly smaller and has no homology with P1 of subgroup II viruses, lacks a VPg that is homologous with that of subgroup II (van der Wilk et al., 1997) and, in addition, also lacks a serine proteinase motif (Mayo & Ziegler-Graff, 1996). P1-C25 has nucleic acid binding properties, suggesting that it may form complexes with PLRV genomic RNA to facilitate the covalent binding of VPg to the 5′ terminus of PLRV genomic RNA.

We have previously demonstrated the utility of the baculovirus protein expression system for studying aspects of PLRV biology by expressing virus-like particles (VLP) in insect cells (Lamb et al., 1996). In this paper, we report the expression of P1 protein in insect cells and demonstrate that, in these cells, P1 is processed in a manner similar to that observed in planta, that P1 is responsible for this proteolytic activity, and that the chymotrypsin-like serine proteinase motif of P1 is responsible for the observed cleavage.

Methods

The numbering scheme of amino acids referred to below is that of van der Wilk et al. (1997).

Construction of plasmids

pSAB53. This plasmid contains the full-length PLRV genome inserted into vector pUC19 with the insertion of a T7 RNA polymerase promoter sequence immediately upstream of the 5′ terminus of the PLRV sequences. Details as to the construction of this plasmid will be presented elsewhere (F. E. Gildow and others, unpublished results). We were unable to rescue virus back from this construct and subsequent nucleotide sequencing studies showed a point mutation producing a coding change (G

were present in P1 was corrected by site-directed mutagenesis. Sequences encoding the N-terminal half of the P1 protein were amplified using oligonucleotide primers 304 (5′ GACTGATCTCCACATGACAGATTACCCGATAG 3′) and 208 (5′ GTTTTCCGGAATATCC 3′), the reverse primer 208 correcting the point mutation. The sequences encoding the C-terminal half of P1 were amplified using primers 3329 (5′ GACCCGGATATTCGCGAGGTTT GAGTTTACCCGATAG 3′) and 305 (5′ ACTGGAATTTCAGCTTTTGGAGTTTACCCGATAG 3′), the forward primer 3329 (overlapping primer 208) also encoding the correction. In the former case, the PCR product was restricted with BglII and AccI, whereas in the latter case, the PCR product was restricted with Accl and EcoRI. The purified restriction fragments were ligated together with the vector pGEM3zf(+) (Promega) restricted with BamHI and EcoRI.

pVLP1GVpK. Sequences encoding the entire P1 region of pSAB53 were amplified using primers 304 and 155 (5′ ACTGGAGCTCGGCTTTGGAGTTTACCCGATAG 3′). The PCR product was restricted with HindIII, then doubly restricted with either BglII or SacI. The BglII–HindIII and HindIII–SacI restriction fragments were ligated together with plasmid pVL140P (Hakne et al., 1995) restricted with BamHI and SacI. This three-way ligation strategy was adopted due to the presence of an internal SacI restriction enzyme site. This plasmid encodes, therefore, the P1 region (G

pHP1XP. Plasmid pSAB53 was doubly restricted with Xhol and PstI. The Xhol–PstI restriction fragment encoding most of the PLRV P1 protein (see Fig. 1) was purified and ligated into the E. coli expression vector pRSETb, which was similarly restricted.

pGEX-P1SP. Plasmid pHP1XP was restricted with Smal and EcoRI and the restriction fragment encoding the C-terminal region of PLRV P1 (delimited by the Smal and PstI sites within P1) was purified. This fragment was ligated together with the E. coli expression vector pGEX-KT, which was similarly restricted.

pBacP1. Sequences encoding the P1 region were amplified by PCR using the template plasmid pXL1 and primers 304 and 305. The PCR product was restricted with BglII and EcoRI, and ligated into the vector pFastBac (GibcoBRL), which was similarly restricted.

pBacP1PK. Plasmid pVLP1GVpK was initially restricted with KpnI (present in the vector sequences downstream of the insert) and subsequently Smal. The small restriction fragment (encoding sequences downstream of the proteinase domain together with the Pk tag) was purified and ligated with the vector pBacP1, which was similarly restricted.

pBacP1GV and pBacP1GVpK. These plasmids were obtained similarly to pBacP1 and pBacP1PK except that instead of using pXL1 as PCR template, pSAB53 was used, which contained the G

Plasmid pVLP1GVPK was ligated into the vector pVL1393 (Invitrogen) restricted with BglII, SmaI and PstI. The 5·8 kb restriction fragment, which contained the whole PLRV genome, was ligated into the vector pVL1393 (Invitrogen) restricted with Smal and BglII.
Table 1. Oligonucleotides and cloning strategies used in the generation of site-directed P1 mutants

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Primer 3</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBacP1–2</td>
<td>H234 → A</td>
<td>aHA</td>
<td>5637</td>
<td>304</td>
<td>XhoI/XbaI</td>
</tr>
<tr>
<td>pBacP1–3</td>
<td>H255 → W</td>
<td>bHW</td>
<td>304</td>
<td></td>
<td>XhoI/XbaI</td>
</tr>
<tr>
<td>pBacP1–4</td>
<td>H255 → A</td>
<td>bHA</td>
<td>304</td>
<td></td>
<td>XhoI/XbaI</td>
</tr>
<tr>
<td>pBacP1–5</td>
<td>D286 → E</td>
<td>5DE</td>
<td>304</td>
<td>5637</td>
<td>XhoI/StoI</td>
</tr>
<tr>
<td>pBacP1–6</td>
<td>D286 → A</td>
<td>eDA</td>
<td>304</td>
<td>5637</td>
<td>XhoI/XbaI</td>
</tr>
<tr>
<td>pBacP1–7</td>
<td>S354 → C</td>
<td>7SC</td>
<td>5637</td>
<td>304</td>
<td>XhoI/StoI</td>
</tr>
<tr>
<td>pBacP1–8</td>
<td>S354 → A</td>
<td>8SA</td>
<td>5637</td>
<td>304</td>
<td>XhoI/StoI</td>
</tr>
<tr>
<td>pBacP1–9</td>
<td>S593 → A</td>
<td>9SA</td>
<td>5637</td>
<td>304</td>
<td>XhoI/StoI</td>
</tr>
</tbody>
</table>

pBacTVAX. Plasmid pVLTV was restricted with XhoI and then recircularized (removing the majority of P1 and P2 sequences).

pGEMP1. The whole P1 sequence was amplified with oligonucleotide primers 304 and 305 using plasmid pXL1 as a template and the PCR product was ligated into the transcription vector pGEM-T easy (Promega).

Site-directed mutagenesis. A series of mutations were introduced into the putative protease domain by PCR; plasmid pBacP1 was used as template. Five conserved amino acids, three of which comprise the proposed triad of H255, D286 and S354, were mutated. Details of these constructs are shown in Table 1. Briefly, primers 1 and 2 were used in the first-round PCR. The amplified product was then used as a primer in the second round of amplification, along with primer 3. The amplified product from second-round PCR was then restricted and ligated into pBacP1, which was similarly restricted. For pBacP1–3 and pBacP1–4, the products of the first-round PCR were cut with XhoI and XbaI, and ligated into similarly restricted pBacP1. The resulting ‘donor’ plasmids (Table 1, column 1) were used to generate recombinant baculovirus. The sequences of the oligonucleotides used in the generation of these mutants (and referred to in Table 1) are: 5′-TAAAG-GCTTGGTTGTTAG-3′; aHA, 5′-GAAAAGGCCGACGCTCGG-GTAC-3′; bHW, 5′-CTTCTAGACACATTCAAGTGTACCC-3′; bHA, 5′-CTTCTTAGACATGCTCGTGTTACC-3′; 5DE, 5′-TT- CATTACGGGACCTTGGT-3′; eDA, 5′-AGGATTACGGGCACCTT- TTG-3′; 7SC, 5′-TGGCGGAACAGGGTTTTGCT-3′; 8SA, 5′-GCCG-GGAACAGGGTTTTGCT-3′; 9SA, 5′-GCCGAAAATATGTT-TGA-3′.

Construction of recombinant baculoviruses. Baculovirus BacTVAX was generated by co-transfection with Bsu36I-cleaved viral DNA (Possee & Howard, 1987). Other recombinant baculoviruses were constructed using the Bac-to-Bac baculovirus expression system (GibcoBRL). The recombinant donor plasmids were used to transform E. coli strain DH10B, which contained bacmid BMON14272 and the helper plasmid pMON7124. White colonies, which harbour the recombinant bacmids generated by transposition, were selected. Recombinant bacmids were purified from E. coli DH10B and used to transfect SF9 cells to generate the corresponding baculoviruses.

Preparation of polyclonal antiserum. E. coli JM101 containing plasmid pGEX-P1SP was induced at OD600 of 1.0 with IPTG (0.4 mM) and cells were harvested 4 h later. The fusion protein GST–P1SP was purified using glutathione agarose beads, and cleaved with thrombin while still bound to the beads. The released protein, P1SP, was mixed with complete Freund’s adjuvant and used to immunize rabbits. Booster injections (100 µg antigen) were administered at 2 week intervals using incomplete Freund’s adjuvant. The titres were monitored by ELISA 10 days after each booster. The antisera collected after the fourth booster (500 µg antigen) were tested by ELISA and Western blotting and used in this study. Mouse monoclonal anti-Pk tag antibodies were a kind gift of R. E. Randall.

Tissue culture and baculovirus propagation. Procedures used in virus purification, amplification and expression of PLRV proteins in SF9 insect cells were performed as described previously (O’Reilly et al., 1992). For co-infection experiments, appropriate volumes of two virus stocks were mixed and used as an inoculum.

Western blotting. Infected SF9 cells were harvested at the times indicated, washed with PBS and boiled after adding SDS loading buffer. Proteins were resolved by SDS–PAGE (10% or 13%) and transferred onto a PVDF membrane as described previously (Bjerrum & Schafer-Nielson, 1986). Membranes were blocked with PBS/0·1% Tween 20/0·1% BSA/0·1% Ficoll/0·1% PVP for 4 h at room temperature or overnight at 4 °C. The rabbit polyclonal anti-P1SP primary antibodies (1:2000 dilution in blocking buffer) were incubated with the membranes for 1 h. The mouse monoclonal anti-Pk tag antibodies (1:1000 dilution in blocking buffer) were incubated with the membranes for 1 h. Membranes were then washed with PBS/0·1% Tween 20 and incubated for 1 h with peroxidase-conjugated goat anti-rabbit or mouse IgG (1:4000 dilution in blocking buffer). Bound secondary antibody was detected using enhanced chemiluminescence (Amersham).

Transmission/translation in vitro. A coupled transcription/translation wheatgerm expression system (TntT; Promega) was programmed with 1 µg unrestricted pGEMP1 plasmid DNA and incubated at 30 °C for 90 min, in the presence of T7 RNA polymerase and [35S]methionine, as recommended by the manufacturer. Extended incubations were performed following the arrest of translation by the addition of RNase (0·5 mg/ml) and cycloheximide (0·8 mg/ml). Translation products were analysed by SDS–PAGE and autoradiography.

DNA sequencing. The nucleotide sequence of all cDNA clones derived from PCR amplification was confirmed by automated DNA sequencing using a PE Applied Biosystems 377 Prism machine.

Results

Luteovirus protein P1 is proteolytically processed in insect cells

To study the biochemical functions of P1, we constructed two recombinant baculoviruses containing the P1 coding
Serine proteinase domain in PLRV P1 protein

Fig. 2. Western blots of luteovirus P1 proteins. Extracts of Sf9 cells infected with recombinant baculoviruses (2 days post-infection) were resolved by SDS–PAGE (13%), blotted and probed with anti-P1SP antiserum (A), or extracts (3 days post-infection) were resolved by SDS–PAGE (10%), blotted and probed with anti-Pk tag monoclonal antibodies (B).

Fig. 3. P1 self-processing in trans. Extracts of Sf9 cells infected with recombinant baculoviruses (2 days post-infection) were probed with anti-Pk tag monoclonal antibodies. Sf9 cells were infected with either a single type of recombinant baculovirus (lanes 1, 5 and 6), or a mixture of baculoviruses (lanes 2–4). Sf9 extracts produced from mixed infections were from cells infected with BacP1GVPk at m.o.i. of 2, with increasing titres of BacP1 (m.o.i. of 1, 2 and 4 in lanes 2, 3 and 4, respectively).

region whose expression was directed by the baculovirus (Autographa californica nucleopolyhedrovirus) polyhedrin promoter. The recombinant baculovirus BacP1 expressed the native P1 protein, whereas the P1 protein encoded by BacP1Pk had a C-terminal 16 aa extension corresponding to the highly antigenic Pk tag from simian virus 5 (Hanke et al., 1995). The expression of P1 and P1Pk in insect cells was analysed by Western blotting. The predicted molecular masses of P1 and P1Pk are 70 kDa and 71 kDa, respectively. Western blot analyses using anti-P1 antisera showed, however, that both P1 and P1Pk were processed to smaller products (Fig. 2), the C-terminal Pk tag extension having no discernible effect on processing. Western blotting using a monoclonal antibody raised against the Pk tag detected a cleavage product of ~27 kDa. Since the Pk tag is a C-terminal extension of P1, this indicated that the cleavage site is at the N-terminal region of VPg (Fig. 2B).

A single point mutation blocks processing

During construction of the recombinant baculoviruses BacP1 and BacP1Pk, a clone was isolated that had a single point mutation resulting in the coding change G\(^{355}\) → V. This mutation is at a conserved proteinase motif (GxSG), G\(^{355}\) corresponding to the glycine residue predicted to be involved in the formation of the oxyanion ‘hole’ of the putative proteolytic domain. To investigate the effect of the G\(^{355}\) → V mutation, two further recombinant baculoviruses were constructed (BacP1GV and BacP1GVPk), each bearing this point mutation. Western blot analyses of Sf9 cells infected with BacP1GV and BacP1GVPk viruses showed that only full-length proteins were produced (Fig. 2A). These observations are consistent with residue G\(^{355}\) being an important component of the proposed serine proteinase domain (Fig. 1B) and mitigate against the processing we observed with BacP1 and BacP1Pk being due to non-specific degradation.

P1 processing is mediated by P1 itself

Proteolysis of P1 in baculovirus-infected Sf9 cells could be accounted for in two ways: either P1 contains a functional proteolytic domain, or the G\(^{355}\) → V mutation renders P1 not
susceptible to cleavage by an undefined cellular or baculovirus-specific proteinase. To address this question, Sf9 cells were co-infected with BacP1GVPk (Pk-tagged protein P1; not cleaved in this system), together with BacP1, the (untagged) P1 protein which was cleaved. Western blotting with monoclonal antibody directed against the Pk tag showed that protein P1GVPk was cleaved upon co-infection with BacP1 (Fig. 3). Taken together, these data show that protein P1GVPk functioned as a substrate (alone), was processed in trans by the wild-type P1 protein (expressed from BacP1) and not by a cellular or baculovirus-specific proteinase. This conclusion is consistent with other co-infection experiments we have performed. By increasing the m.o.i. ratio of BacP1:BacP1GVPk, increased P1 processing in trans is observed. Similar observations are made with cells singly infected with BacP1; at m.o.i. of 0:1, processing is partial with substantial amounts of uncleaved P1 observed (Fig. 2B), whereas at m.o.i. of 2, processing of P1 appeared to be complete (Fig. 3, lane 1).

**Processing in vitro**

Processing of P1 was also studied in a coupled transcription/translation wheatgerm extract (Fig. 4). Reactions performed for 90 min programmes with plasmid pGEMP1 (encoding the wild-type P1 sequence) showed that the major translation product was the full-length P1 protein (70 kDa; Fig. 4, lane 1). Translation reactions programmed with a similar construct containing the C$^{335}$V mutation produced an identical translation profile (data not shown). The minor translation products observed were, therefore, due to internal initiation. In each case, we were unable to observe proteolytic processing, either in cis or in trans. Extended incubations (following the arrest of translation), however, showed the appearance of two new products of $\sim 43$ kDa and $\sim 27$ kDa (Fig. 4, lane 2). The 27 kDa product corresponds with the product observed by Western blotting of the baculovirus extracts with the anti-P1SP antiserum, which recognizes the region of P1 indicated in Fig. 1.

**Characterization of the P1 proteolytic domain**

The presence of a chymotrypsin-like proteolytic domain within the luteovirus subgroup II P1 proteins has long been suspected and a catalytic triad has been identified, but not proven (Gorbalenya et al., 1989; Miller et al., 1995). To confirm that the proposed P1 proteolytic domain was functional and responsible for the cleavage, we mutared five P1 amino acids at positions which are completely conserved among subgroup II luteoviruses (poleroviruses), some of which are proposed to be essential for proteolytic activity (Fig. 1B). Mutation of the conserved residues H$^{234}$ and Ser$^{393}$ produced a proteolytically active enzyme; the 27 kDa cleavage product was observed, although in both cases an additional $\sim 50$ kDa product was detected (Fig. 5). Following mutation of the residues proposed to form the catalytic triad (H$^{234}$ → W/A, D$^{286}$ → E/A, S$^{344}$ → C/A), the proteolytic activity of all mutant P1 proteins was completely abolished; only the full-length (mutant) P1 translation product was observed.

**Discussion**

In this paper, we have shown that, in insect cells, P1 is processed in a similar manner to that observed in plant cells. Moreover, we have proved that the observed cleavage is due to P1 proteinase activity, and that the serine proteinase motif is indeed responsible. Although we have not proved it conclusively, our data on the size of the C-terminal cleavage

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**Fig. 4.** Translation in vitro. Coupled transcription/translation reactions were programmed with plasmid pGEMP1 and incubated for 90 min (lane 1), or the translation was arrested and subsequently incubated for an additional 2 h (lane 2). The positions of P1 (70 kDa) and the cleavage products (43 kDa and 27 kDa) are indicated.

**Fig. 5.** Analysis of P1 site-directed mutants. Extracts of Sf9 cells infected with recombinant baculoviruses encoding mutant forms of the P1 proteinase domain were probed with anti-P1SP antiserum.
products would support the notion that the P1 cleavage we observed was at, or near, the N terminus of VPg. Experiments which have determined the size of VPg attached to the viral RNA indicate this protein is some 8 kDa (Mayo et al., 1982), from which one may assume an additional cleavage to form the C terminus of VPg. At present, we have no evidence for a second cleavage of the C terminus of P1 in the baculovirus system, although it should be noted that the 5′ end of the PLRV genomic RNA was not present.

A PLRV P1 C-terminal, hydrophilic, basic region containing the sequence KxKKxRRxxRxK separate from the VPg region and within the terminal 25 kDa (encoded by nt 1848–1892) has been shown to bind labelled PLRV RNA (Prüfer et al., 1999). This has led this group to propose a model for VPg maturation in which C25 (containing VPg) is cleaved by the viral proteinase from the N-terminal region of P1. This region contains the viral proteinase and a hydrophobic region which they propose is membrane-bound. The hydrophilic C terminus, including the VPg and PLRV RNA binding region, is bound to genomic RNA and the VPg is transferred from C25 to the 5′ end of the genomic RNA by some unspecified mechanism. Our data support the first part of this model if not (yet) the second.

Unexpectedly, using in vitro translation systems – both wheat germ extracts (this paper) and rabbit reticulocyte lysates (data not shown) – we were unable to demonstrate a proteinase activity in cis, although we were able to detect some processing in trans upon extended incubation (2 h). In contrast, in the expression of the identical ORF in vivo, in which expressed products were present at much higher concentrations, proteolytic processing was observed. We also observed that the processing of P1 occurred more rapidly in insect cells infected at high m.o.i. (m.o.i. of 2) than those infected at low (~0·1) m.o.i.

Currently, we have no evidence for cis (intramolecular) cleavage. These data may be interpreted in two ways: firstly, that the proteinase cleaves P1 in trans and not in cis, or secondly, that the proteinase requires activation by a co-factor (supplied in trans) that would be unable to activate the proteinase due to the extremely low concentration of products generated in such cell-free translation systems. If such a cofactor is required we can conclude that: (i) if such a factor is viral in origin, this factor is present within P1 since processing was observed in vivo with only P1 luteovirus sequences present; and (ii) if it is cellular in origin, then it is present in both insect and plant cells.

Whether this cleavage reaction is related to the unusual position of the VPg downstream rather than upstream of the viral proteinase domain remains to be determined. A mechanism of cleavage in trans implies an interaction between two (or more) P1 molecules to bring about cleavage. The presence of N-terminal hydrophobic domains (thought to be involved in membrane binding) within P1 may have a significant role both in the cellular localization and kinetics of P1 association.

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**References**


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