Expression of potyvirus proteins in insect cells infected with a recombinant baculovirus

D. W. Thornbury, J. F. J. M. van den Heuvel, J. A. Lesnaw and T. P. Pirone

Departments of Plant Pathology and Biological Sciences, University of Kentucky, Lexington, Kentucky 40546, U.S.A.

The N-terminal portion (P1–HC-Pro–P3) of the tobacco vein mottling virus (TVMV) polyprotein was expressed in insect cells and larvae by a recombinant baculovirus. The proteases necessary to process this TVMV polyprotein fragment were active in insect cells, since mature P1, HC-Pro and P3 proteins were detected by specific antisera in Western blots. Antisera to P1, HC-Pro and P3 also recognized polypeptides with apparent Mₚ values predicted for the intermediate processing products of the polyprotein fragment. The results of this study indicate that the autocatalytic processing of TVMV HC-Pro from the polyprotein is supported by insect cells. Helper component activity in extracts of cells infected with recombinant baculovirus was not detected by aphid transmission assay.

The potyviruses are filamentous, positive-strand RNA plant viruses which have an approximately 10 kb genome encoding a polyprotein which is proteolytically processed into at least eight products (Dougherty & Carrington, 1988). The N-terminal third of the genome codes for three proteins, P1, HC-Pro and P3, each of which have been detected in tobacco vein mottling virus (TVMV)-infected plants (Thornbury et al., 1985; Rodriguez-Cerezo & Shaw, 1991). In tobacco etch virus (TEV) two of these proteins, HC-Pro and P1 (35K), contain protease domains which are responsible for their release from the polyprotein (Carrington et al., 1989; Verchot et al., 1991). The N-terminal portion of the HC-Pro protein contains the helper component domain necessary for transmission of potyviruses by aphids (Thornbury et al., 1985; Atreya et al., 1992) and approximately 20K of the C-terminal portion contains the proteolytic domain which cleaves between a Gly–Gly dipeptide located between HC-Pro and P3 (Carrington et al., 1989). Verchot et al. (1991) showed in wheat germ cell-free translations that the N terminus of TEV HC-Pro is cleaved by a protease domain at the C terminus of the TEV P1 protein.

Potyvirus proteins have been expressed in cell-free translation systems, Escherichia coli, and transgenic plants. Cell-free translation systems have been used to identify and map the potyvirus genes and gene products (Dougherty & Hiebert, 1980; Hellmann et al., 1984), and to characterize the proteases involved in processing the polyprotein (Carrington & Dougherty, 1987; Carrington et al., 1988, 1989; Hellmann et al., 1988; Verchot et al., 1991). E. coli expression systems have been used to produce polypeptides for generation of antisera to coat protein and NIb (Nagel & Hiebert, 1985), TEV HC-Pro (Carrington et al., 1990), TVMV P1 (34K) and P3 (42K) (Rodriguez-Cerezo & Shaw, 1991), and to study processing by the NIa protease (Garcia et al., 1989; LaLiberté et al., 1992). Transgenic plant systems support proper processing of the polyprotein and produce biologically active HC-Pro (Berger et al., 1989; Carrington et al., 1990).

Expression of potyvirus proteins in the baculovirus expression vector system (BEVS) is an attractive alternative to bacterial and cell-free systems because insect cells support post-translational modifications in a manner similar to other eukaryotic systems and are thus capable of producing authentic proteins (Maeda, 1983; Hiebert et al., 1984), and to characterize the proteases involved in processing the polyprotein (Carrington & Dougherty, 1987; Carrington et al., 1988, 1989; Hellmann et al., 1988; Verchot et al., 1991). E. coli expression systems have been used to produce polypeptides for generation of antisera to coat protein and NIb (Nagel & Hiebert, 1985), TEV HC-Pro (Carrington et al., 1990), TVMV P1 (34K) and P3 (42K) (Rodriguez-Cerezo & Shaw, 1991), and to study processing by the NIa protease (Garcia et al., 1989; LaLiberté et al., 1992). Transgenic plant systems support proper processing of the polyprotein and produce biologically active HC-Pro (Berger et al., 1989; Carrington et al., 1990).

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Production of recombinant baculovirus expressing potyvirus proteins was mediated using the transfer vector pJVP10Z, kindly provided by Dr C. Richardson (Biotechnology Research Institute, Montreal, Quebec, Canada). This vector contains two promoters derived
from the P10 and polyhedrin genes of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Vialard et al., 1990). The P10 promoter directs synthesis of β-galactosidase as a reporter gene to identify recombinants. Expression of potyvirus genes under the control of the polyhedrin promoter is achieved by insertion of constructs into an *NheI* site 3' to the promoter.

A construct containing bases 206 to 3541 of the TVMV genome was prepared from cDNA clones using PCR and specific oligonucleotides which introduced *XbaI* sites at the 5' and 3' ends and an in-frame termination codon after base 3541. The construct included the initiation codon of the TVMV polyprotein and the first three cistrons (P1–HC-Pro–P3) of the viral genome. After digestion with *XbaI* the 3348 bp fragment was cloned into the compatible *NheI* site of pJVP10Z to generate pTHC1OZ. The plasmid was CsCl-purified from *E. coli* strain DH5α and the fidelity of the PCR was confirmed by sequencing.

*Spodoptera frugiperda* (SF9) cells (Invitrogen), were cultured in Grace's Antheraea Medium (GAM) plus supplements and 10% fetal bovine serum (FBS) according to the manufacturer's recommendations. Cells were cotransfected with the DNA from the plasmid and wild-type AcMNPV DNA (Invitrogen) by the calcium phosphate precipitation method (Carstens et al., 1980) followed by a glycerol shock, using 20% glycerol in GAM for 1 min at room temperature. Recombinant plaques were identified by overlay of plaque assay plates with 0.5% X-Gal in agarose, as α-β-galactosidase activity indicator (Vialard et al., 1990). Recombinant plaques were found to be free of polyhedra after two or three rounds of plaque purification. Proteins from recombinant plaques were compared on SDS–polyacrylamide gels with those from wild-type AcMNPV plaques and were found to be free of the polyhedrin protein.

Baculovirus from plaques positive for β-galactosidase was used to infect 2.5 × 10^6 SF9 cells following the procedure described by Summers & Smith (1987). Cells were harvested 2 to 4 days post-infection (p.i.) and assayed for expression of potyvirus HC-Pro by SDS–PAGE in 10% acrylamide gels (Laemmli, 1970) and Western blot analysis (Towbin et al., 1979) using antiserum to TVMV HC-Pro (Thornbury et al., 1985). Enrichment stocks of recombinant baculovirus were made and the virus titre was determined in plaque assays as described by Summers & Smith (1987).

The expression of potyvirus peptides in insect cells was followed after inoculation of SF9 or *Trichoplusia ni* cells (High Five cells, Invitrogen) with the recombinant baculovirus at an m.o.i. of 10. Cultures of both cell lines (3 × 10^6 cells) were inoculated with the recombinant baculovirus at an m.o.i. of 10. Two days p.i., cells were collected, washed to remove FBS and resuspended in 3 ml of 100 mM-Tris–H₂SO₄ pH 7.2, 20 mM-MgSO₄ (TSM). Cells were then lysed by sonication and centrifuged at 16000 g. Aliquots of pellet and supernatant fractions from equal numbers of cells were compared by Western blot analysis using antisera to TVMV HC-Pro (Fig. 1). Four major products with *M*ₜ of 126K, 97K, 81K and 52K were detected in supernatant and pellet fractions (Fig. 1, lanes 1 and 2) of High Five cells; the latter were used in the remainder of this study.

Identification of the 126K, 97K and 81K bands was achieved using antisera to TVMV P1 and P3 polypeptides produced in bacteria (Rodríguez-Cerezo & Shaw, 1991) in addition to HC-Pro antiserum. Four days p.i. 3 × 10^6 cells infected with the recombinant baculovirus were collected and analysed by Western blot. Cell pellets, washed as before, were resuspended in 500 μl of TSM, diluted with an equal volume of 4 × Laemmli loading buffer and heated for 5 min at 100 °C. Antiseras to TVMV P1 and P3 detected polypeptides of the expected size (Fig. 2, lane 4, 34K; lane 2, 42K). Antiseras to P1 also recognized the 81K and 126K polypeptides (lane 1) detected by antiseras to TVMV HC-Pro, and antiseras to P3 protein recognized the 97K and 126K products (lane 2). The observation that an 81K product is recognized by both anti-P1 and anti-HC-Pro sera and that a 97K protein reacts with antiseras to both P3 and HC-Pro suggests that these polypeptides are intermediate products of polyprotein processing by the protease domains...
of the TVMV P1 and HC-Pro proteins. The fact that more of the 81K product than the 97K product is detected in lanes treated with either HC-Pro, P1 or P3 antiserum suggests that the HC-Pro/P3 junction is more efficiently cleaved than the P1/HC-Pro junction. This is also supported by the fact that more P3 is present in lane 2 than P1 in lane 1. Verchot et al. (1992) suggested that the efficiency of these protease domains may provide temporal regulation of the release of proteins from the polyprotein; the BEVS would provide a useful system to study the order of release of potyvirus proteins. Since the processing of the P1/HC-Pro junction appears to be relatively slow, it is possible that the 81K protein may also have a biological function as well as being a precursor of P1 and HC-Pro.

HC-Pro must be in a soluble form in order to have helper component activity in the aphid bioassay (D. W. Thornbury, unpublished data). Most of the HC-Pro and other potyvirus polypeptides expressed by recombinant baculovirus were consistently found in insoluble fractions of cell extracts, and attempts to prepare extracts having helper component activity from insect cells using procedures used for plant-derived HC-Pro (Thornbury et al., 1985) were unsuccessful. Espinoza et al. (1992) and Blanc et al. (1993) reported similar problems with extraction of cauliflower mosaic virus helper component (CaMV aphid transmission factor, ATF) from insect cells infected with recombinant baculovirus. However, Blanc et al. (1993) were able to recover active CaMV ATF expressed by recombinant baculovirus by an extraction procedure which was least destructive of the native structure of the expressed protein. Their procedure, which is similar to that used for purification of CaMV ATF from plants, was not effective for recovery of TVMV helper component activity expressed in insect cells (data not shown).

An attempt was made to develop procedures for solubilization of HC-Pro having helper component activity. A number of procedures based on solubilization by chaotropic agents followed by gradual renaturation were first tested on active helper component which had been partially purified from infected tobacco plants. Urea, guanidine–HCl or the zwitterionic detergent CHAPS each in 2 x TSM were tested (Table 1). Concentrated TVMV HC-Pro was diluted 10-fold in each solution and incubated for 1 hour at 4 °C, dialysed 18 h against 2 x TSM, concentrated by precipitation with 8% PEG and resuspended to its original volume in 2 x TSM.

Table 1. The effect of chaotropic agents and the zwitterionic detergent CHAPS on activity of helper component purified from TVMV-infected plants

<table>
<thead>
<tr>
<th>Treatment of HC-Pro*</th>
<th>Aphid transmission of TEV (%)†</th>
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<tbody>
<tr>
<td>Control HC-Pro</td>
<td>80</td>
</tr>
<tr>
<td>0.5 M-urea</td>
<td>80</td>
</tr>
<tr>
<td>1 M-urea</td>
<td>50</td>
</tr>
<tr>
<td>2 M-urea</td>
<td>20</td>
</tr>
<tr>
<td>4 M-urea</td>
<td>0</td>
</tr>
<tr>
<td>6 M-urea</td>
<td>0</td>
</tr>
<tr>
<td>0.5 M-guanidine–HCl</td>
<td>0</td>
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<tr>
<td>1 M-guanidine–HCl</td>
<td>0</td>
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<tr>
<td>2 M-guanidine–HCl</td>
<td>0</td>
</tr>
<tr>
<td>5 mM-CHAPS</td>
<td>40</td>
</tr>
<tr>
<td>20 mM-CHAPS</td>
<td>20</td>
</tr>
<tr>
<td>5 mM-CHAPS + 0.5 M-urea</td>
<td>50</td>
</tr>
<tr>
<td>20 mM-CHAPS + 0.5 M-urea</td>
<td>30</td>
</tr>
</tbody>
</table>

* Treatments consisted of chaotropic agents or CHAPS dissolved in 2 x TSM. TVMV HC-Pro purified from plants was diluted 1/10 in each treatment, incubated 1.5 h at 4 °C, dialysed 18 h against 2 x TSM, concentrated by precipitation with 8% PEG and resuspended to its original volume in 2 x TSM.
† Percentage of infected plants; 10 aphids were placed on each of 10 test plants for each treatment.
and CHAPS as low as 5 mM resulted in a 25 to 60% reduction helper component activity as compared to the control HC-Pro (Table 1). Extraction procedures used for plant tissue supplemented with the treatments in Table 1 that did not destroy helper component activity were tested for recovery of TVMV HC-Pro expressed in insect cells. In all cases HC-Pro was found only in pellet fractions and no fractions contained helper component activity detectable by aphid bioassay (data not shown). The active form of potyvirus helper component recovered from virus-infected plants is probably a dimer of the 52K HC-Pro protein (Thornbury et al., 1985), and biological activity may depend upon proper folding during translation and/or proper subunit association during polyprotein processing. Our experiments with helper component from infected plants in the present study indicate that once the structure necessary for transmission activity is destroyed it cannot be recovered.

The TVMV gene products expressed by a recombinant baculovirus appear to have been processed properly and to have undergone the post-translational modifications necessary to produce proteins with the same apparent Mr as those produced in tobacco plants infected with TVMV. The protease responsible for cleavage of the P1/HC-Pro junction and the protease domain of HC-Pro were active in insect cells and produced HC-Pro protein that comigrated with authentic HC-Pro from plants. P1 and P3 proteins having the correct Mr were also produced in the insect cells. These results extend to TVMV the observation of Verchot et al. (1991) that the TEV P1 protein contains the protease that cleaves the P1/HC-Pro junction in the TEV polyprotein.

Although the recovery of helper component activity from the BEVS has been thus far unsuccessful the results of these studies suggest that proper post-translational processing of potyvirus proteins occurs in insect cells. The expression of potyvirus genes in the BEVS may be useful for study of the regulation of polyprotein processing and determining the unknown functions of proteins such as P1 and P3.

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