Processing and subcellular localization of the leader papain-like proteinase of Beet yellows closterovirus

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ORF 1a of Beet yellows closterovirus (BYV) encodes the domains of the papain-like proteinase (PCP), methyltransferase (MT) and RNA helicase. BYV cDNA inserts encoding the PCP–MT region were cloned in pGEX vectors next to the glutathione S-transferase gene (GST). In a ‘double tag’ construct, the GST–PCP–MT cDNA was flanked by the 3′-terminal six histidine triplets. Following expression in E. coli, the fusion proteins were specifically self-cleaved into the GST–PCP and MT fragments. MT–His6 was purified on Ni–NTA agarose and its N-terminal sequence determined by Edman degradation as GVEEEA, thus providing direct evidence for the Gly588/Gly589 bond cleavage. The GST–PCP fragment purified on glutathione S–agarose was used as an immunogen to produce anti-PCP monoclonal antibodies (mAbs). On Western blots of proteins from virus-infected Tetragonia expansa, the mAbs recognized the 66 kDa protein. Immunogold labelling of BYV-infected tissue clearly indicated association of the PCP with the BYV-induced membranous vesicle aggregates, structures related to closterovirus replication.
cleavage in a cell-free translation system, thus providing experimental support, albeit circumstantial, to the identification of the scissile bond (Agranovsky et al., 1994). Although BYV L-PCP and potyvirus HC-Pro show no similarity apart from the ~140-residue PCP domains, both are multifunctional proteins with common activities. Like HC-Pro, BYV L-PCP influences virus RNA amplification and cell-to-cell movement (Peremyslov et al., 1998; Peng & Dolja, 2000; Peng et al., 2003).

In this study, we wanted to gain more insight into the processing and subcellular localization of BYV L-PCP. Bacterial expression vectors were constructed, which allowed us to express L-PCP fusion proteins, monitor their self-cleavage and purify the N- and C-terminal cleavage products for microsequencing and for mAb production.

For L-PCP cloning, the insert from the BYV cDNA clone 154 (Agranovsky et al., 1994) was excised with EcoRI/Eco52I and ligated between the same sites of pGEX-4T3 (Amersham Pharmacia Biotech). The EcoRI–Eco72I fragment of the BYV 1518 clone was inserted between the EcoRI and SmaI sites of pGEX-4T1. The resulting clones, pGEX-1518 and pGEX-154, carried the glutathione S-transferase (GST) gene fused in frame with portions of the BYV ORF1a encoding, respectively, aa 259–720 and aa 302–683 (BYV 1a numbering: Fig. 1). To produce pGEX-1518Δmt (encoding a C-terminally truncated protein, aa 259–600), pGEX-1518 was digested with BspTI and Eco52I, treated with Klenow fragment and religated. Plasmid pGEX-1518Cys 509 was constructed by replacing an Eco47III–Eco52I fragment in pGEX-1518 with the same fragment from pB515C509 (containing the point substitution Cys 509/Thr) (Agranovsky et al., 1994). To obtain a BYV cDNA flanked by the 5′-terminal GST gene and the 3′-terminal six histidine triplets (pGEX-1518-His6), the XhoI–HindIII fragment from the pQE-p65-C6H vector (Agranovsky et al., 1997) was inserted between the SalI and NcoI sites of pGEX-1518. The plasmids were used for transformation of the E. coli strain BL-21. The following conditions were found to be optimal for bacterial expression of BYV L-PCP fusion proteins: E. coli cultures were allowed to grow to OD600 = 0.6 at 33 °C, followed by induction with 0.2 mM IPTG and further growth for 4 h at 26 °C.

The IPTG-induced cells harbouring pGEX-154 accumulated proteins migrating as 55 and 70 kDa entities, which were absent from the non-induced control (Fig. 2A, lanes 1 and 2). Likewise, the 62 and 76 kDa proteins accumulated in the induced pGEX-1518-containing cells (Fig. 2A, lane 3). The single 62 or 76 kDa products were detected in the cells harbouring pGEX-1518Δmt (cDNA with a deletion of the MT sequence) or pGEX-1518Cys509 (cDNA with a mutation of catalytic Cys509 abolishing the PCP activity in vitro; Agranovsky et al., 1994) (Fig. 2A, lanes 4 and 5). The sizes of the 55 and 62 kDa proteins were consistent with self-processing of the BYV BYV PCP in bacteria transformed by the respective pGEX-154 or pGEX-1518 vectors, whereas the 70 and 76 kDa proteins apparently represented uncleaved products. The respective C-terminal 10 and 15 kDa cleavage products could not be confidently identified, presumably because of their instability and/or masking by the abundant low molecular mass E. coli proteins (Fig. 2A).

Following affinity purification on glutathione S–agarose (Smith & Johnson, 1988), a single 62 kDa protein band was detected in the bound-and-eluted fractions from both the pGEX-1518- and pGEX-1518Δmt-transformed cell extracts (Fig. 2A, lanes 6 and 7). The absence of the uncleaved 76 kDa protein in the bound fraction of the GST–1518 proteins (Fig. 2A, lane 6) may be explained by continued self-processing of the protein following chromatography in native conditions.

We wanted to identify the BYV L-PCP cleavage site directly by microsequencing the N terminus of an MT-containing cleavage product. The pGEX-1518-His6 vector was constructed encoding a fusion of GST, PCP–MT and the C-terminal portion of the BYV p65 protein with the His6

![Fig. 1. Schematic representation of the BYV ORF 1a (numbers on the upper scale, triplets from the 5′ end) and the expressed cDNA inserts in the pGEX clones. The glutathione S-transferase (GST) gene and the BYV-specific inserts are shown as grey and white areas within each box, respectively, with the calculated molecular masses of the encoded polypeptides indicated. Insert pGEX-1518Cys509 contained a point mutation of the catalytic cysteine (black diamond). Insert pGEX-1518-His6 encoded an extra tag consisting of the 15 kDa portion of the BYV p65 protein histidylated at the C terminus (p65C-His6; cross-hatched area). The conserved domains are the papain-like proteinase (PCP), methyltransferase (MT) and RNA helicase (HEL). The PCP cleavage site is denoted by a broken line. Drawn approximately to scale.](image-url)
tag (Fig. 1). *E. coli* BL-21 cells containing pGEX-1518-His<sub>6</sub> accumulated proteins of 90, 62 and 28 kDa, whose apparent molecular masses were consistent with those calculated for the respective uncleaved fusion, the N-terminal GST–PCP fragment and the C-terminal MT–p65 fragment (Fig. 1; Fig. 2B, lane 2). In line with this, the protein fraction purified on Ni–NTA agarose in denaturing conditions (Agranovsky et al., 1997), contained only the C-terminal 28 kDa fragment and the 90 kDa uncleaved protein (Fig. 2B, lane 3). Following transfer to membrane, the 28 kDa protein band was excised and subjected to automated Edman degradation. The N-terminal sequence of the protein fragment was determined as Gly-Val-Asp-Asp-Asp-Ala, thus confirming the BYV PCP cleavage of the Gly<sup>588</sup>/Gly<sup>589</sup> bond in the 1a polyprotein.

Immunization of mice with GST–1518<br>D<sub>mt</sub>, the fusion protein purified from the pGEX-1518<br>D<sub>mt</sub>-transformed *E. coli* (Fig. 2A), and screening of hybridomas by indirect ELISA resulted in five clones (4A1, 4A2, 2B3, 1C3 and 3C1) reacting positively with the recombinant immunogen but not with GST. All five mAbs recognized the GST–1518Am<sub>t</sub> protein on Western blots of total protein from the IPTG-induced cells (data not shown). On Western blot analysis of total phenol-extracted protein from infected *Tetragonia expansa* plants, mAbs 4A1, 4A2, 2B3 and 3C1 recognized the major 66 kDa protein (Fig. 2C), whose apparent size agrees with that of BYV L-PCP released in vitro (Agranovsky et al., 1994) and with the established cleavage site (Fig. 2B). This result was not unexpected, yet was important, as the possibility of additional cleavages within the leader protein had not been excluded — especially in view of the fact that the 1a polyprotein processing in vivo appears to be more sophisticated than it seemed (Erokhina et al., 2000).

In immunogold labelling, all five mAbs reacted with the BYV-induced vesicle aggregates in the infected *T. expansa* cell sections (Fig. 3A and Table 1). Most of the gold label was observed on the membranes and cytoplasm strands separating the vesicle clusters (Fig. 3B). The labelling was statistically significant for the specimens embedded in
Fig. 3. Immunogold labelling and electron microscopy of the BYV-induced vesicle aggregates in the virus-infected Tetragonia expansa tissue cuts embedded in Lowicryl. Labelling was carried out with the PCP panel mAb 1C3 as primary antibody. (A) Overview showing a specifically labelled vesicle aggregate (VA) in contrast to unlabelled virus particle aggregates (VP), chloroplasts (C), mitochondrion (M) and cell wall (W). Bar, 500 nm. (B) Enlarged part of a vesicle aggregate showing specific labelling on the dark membrane/cytoplasm strands (arrows), which surround the unlabelled, low-contrast centres of the vesicle clusters (VC). Bar, 500 nm.
Table 1. Statistics for immunogold labelling of various ultrastructures in healthy and BYV-infected *T. expansa* cells probed with mAbs to the PCP domain of BYV or with heterologous mAbs.

(a) Tissue embedded in Epon

<table>
<thead>
<tr>
<th>mAb</th>
<th>Vesicles</th>
<th>Nuclei</th>
<th>Chloroplasts</th>
<th>Virion aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>3C1</td>
<td>9.5±3.0*</td>
<td>1.9±1.6 (5.3±3.0)</td>
<td>3.0±1.8 (1.0±1.1)</td>
<td>1.0±1.0</td>
</tr>
<tr>
<td>1C3</td>
<td>8.9±2.2</td>
<td>2.1±1.6 (2.4±2.1)</td>
<td>2.9±1.6 (0.9±1.3)</td>
<td>0.4±0.7</td>
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<tr>
<td>2B3</td>
<td>8.7±2.4</td>
<td>1.4±1.2 (2.0±1.6)</td>
<td>3.3±2.2 (0.6±0.7)</td>
<td>0.9±1.1</td>
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<tr>
<td>4A1</td>
<td>21.7±4.1</td>
<td>7.9±3.0 (14.8±7.9)</td>
<td>11.2±4.5 (2.4±1.9)</td>
<td>6.3±4.3</td>
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<tr>
<td>4A2</td>
<td>12.6±2.8</td>
<td>3.8±2.1 (8.8±4.3)</td>
<td>5.1±3.1 (1.9±1.5)</td>
<td>1.8±1.4</td>
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<tr>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
<td>52C1‡</td>
<td>1.3±1.1</td>
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(b) Tissue embedded in Lowicryl

<table>
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<tr>
<th>mAb</th>
<th>Vesicles</th>
<th>Nuclei</th>
<th>Chloroplasts</th>
<th>Virion aggregates</th>
</tr>
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<tbody>
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<tr>
<td>2B3</td>
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<td>5.9±3.1 (3.5±2.3)</td>
<td>1.8±1.2 (2.5±1.8)</td>
<td>1.0±1.7</td>
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<tr>
<td>4A1</td>
<td>21.6±9.4</td>
<td>10.3±4.9 (8.1±3.9)</td>
<td>10.1±3.3 (8.7±3.9)</td>
<td>4.6±4.4</td>
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<tr>
<td>4A2</td>
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<td>2.8±2.1 (4.1±2.4)</td>
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<td>MCA2-4†</td>
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<td>52C1‡</td>
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</table>

*Number of 15 nm gold particles per square unit (4·95 μm²) ± standard deviation, calculated from 20 examined fields. Data in parentheses are counts for similar ultrastructures in healthy cells.
†,‡Specimens treated with mAb MCA2-4 to *Grapevine leafroll-associated closterovirus*-1 or mAb 52C1 to *Wheat streak mosaic tritivirus* as primary antibodies, respectively.

Lowicryl as well as in Epon (Table 1). Specific labelling of similar intensity has been recorded for anti-MT and anti-HEL mAbs (Erokhina *et al.*, 2001). mAbs 4A1 and 4A2 showed an elevated reaction with the nuclei and chloroplasts; however, this was probably non-specific, as a comparable (or higher) labelling was recorded on the healthy tissue sections (Table 1). No mAb reaction with the cell walls and plasmodesmata was observed (data not shown). No labelling of infected cell ultrastructures was seen in the controls with heterologous mAbs (Table 1) or the gold-conjugated secondary antibody alone (not shown).

Taken together, the data of this work clearly show that BYV L-PCP, when expressed in a foreign (prokaryotic) cell context, mediates its specific self-release from a polyprotein. Expression in *E. coli* and affinity purification of the C-terminal cleavage products suitable for microsequencing have previously been reported for human coronavirus 3C-like protease (Ziebuhr & Siddell, 1999) and birnavirus VP4 proteinase (Lejal *et al.*, 2000). We constructed L-PCP fusion proteins with affinity tags at one or both flanks, which allowed purification of the N- and C-terminal cleavage products for antibody production and microsequencing. The pGEX/BL-21 system also obviated the problems encountered in expression of ‘difficult’ proteins, such as BYV L-PCP (Erokhina *et al.*, 2000).

Our electron microscopic analysis of the BYV-infected tissues subjected to immunogold labelling with anti-PCP mAbs indicated association of the leader protein with the closterovirus-induced membranous vesicle aggregates. Replication of positive-strand RNA viruses of animals and plants is connected with vesicles or spherosomes derived from various membranous organelles of the cell (reviewed in Buck, 1996). In Semiliki Forest virus and probably in other Sindbis-like superfamily viruses, the MTR IV motif in the MT domain is responsible for anchoring the replicase complex to membranes (Ahola *et al.*, 1999). On infection of *Brome mosaic bromovirus*, the MT–HEL-containing 1a protein induces budding of the vesicles from endoplasmic reticulum membranes, thus creating secluded replication sites (Schwartz *et al.*, 2002). The BYV methyltransferase-like and helicase-like proteins also reside on the membranes of multivesicular aggregates, thus indicating that these ultrastructures are replication compartments (Erokhina *et al.*, 2001). Co-localization of L-PCP with closterovirus replication-associated proteins agrees with its involvement in RNA accumulation (Peremyslov *et al.*, 1998; Peng &
Dolja, 2000). However, the possibility that the BYV leader protein is also involved in fleeting interactions with other cell compartments and/or virus products to perform activities such as virus long-distance transport (Peng et al., 2003) cannot be excluded.

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