Localization of the P1 protein of potato Y potyvirus in association with cytoplasmic inclusion bodies and in the cytoplasm of infected cells

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The N-terminal P1 proteinase of potato virus Y (ordinary strain group isolate PVY-O) was expressed in E. coli. Antiserum was raised against the expressed protein and used to detect the viral proteins in infected tobacco leaf tissue by Western blotting and by electron microscopy with immunogold labelling. In the immunogold localization studies P1 protein was detected in association with the cytoplasmic inclusion bodies characteristic of PVY infections and in the cytoplasm of the infected plant cells. No significant P1 antibody binding with other plant cell organelles, or with the cell wall and plasmodesmata, was detected by immunogold labelling.

Potato virus Y (PVY) is the type member of the family Potyviridae of positive-sense, single-stranded RNA plant viruses, the largest of the plant virus groups currently known. The potyvirus genome encodes a single large polyprotein that undergoes proteolytic processing, catalysed by virus-encoded proteinases (Dougherty & Selmer, 1993). Functions or putative roles have been assigned to most of the mature viral proteins although additional activities may be associated with polyprotein intermediates (reviewed by Dougherty & Carrington, 1998; Riechmann et al., 1992). However, the role of the N-terminal P1 protein in the virus infection cycle has remained unclear.

The P1 protein is a serine-type proteinase which catalyses autoproteolytic cleavage at a Tyr-Ser dipeptide between itself and the helper component proteinase, HC-Pro (Verchot et al., 1991, 1992). The C-terminal 147 amino acid residues of the P1 protein constitute the complete functional proteinase; the N-terminal 157 amino acid residues are dispensable for proteinase activity (Verchot et al., 1992). In addition to its proteolytic activity, the P1 protein has been shown to exhibit nonspecific RNA-binding activity (Brantley & Hunt, 1993; Soumounou & Laliberte, 1994). The RNA-binding properties of P1 are similar to those described for known movement proteins of plant viruses (Citovsky et al., 1991, 1992; Osman et al., 1992, 1993; Schoumacher et al., 1992) and it has been suggested that P1 could also be involved in cell-to-cell transport of virus in plants (Atabekov & Taliansky, 1990; Brantley & Hunt, 1993; Dougherty & Semler, 1993; Riechmann et al., 1992). However, it has been demonstrated by mutational and complementation analysis for another potyvirus, tobacco etch virus, that the P1 protein plays little, if any, role in virus movement (Verchot & Carrington, 1995a, b). Deletion of the entire P1 coding sequence had only minor effects on cell-to-cell and long-distance transport but considerably reduced genome amplification of TEV mutants, suggesting that the function of P1 is related to virus replication. To further analyse the role of P1 in the life-cycle of potyviruses we investigated the subcellular localization of the PVY P1 protein in infected plant cells.

For antiserum production, the P1 gene from PVY-O was expressed in E. coli. Cloning of the P1 gene has been described by Pehu et al. (1995). The cloned P1 sequence was excised by NdeI and BamHI and ligated into the pET3a expression vector. The construct was transformed into E. coli BL21(DE3)pLysE cells (Novagen). Cell cultures in the exponential growth phase were induced by adding IPTG to a final concentration of 1 mM, and grown for an additional 2.5 h. The cells were pelleted and lysed by the boiling method (Sambrook et al., 1989) for analysis by SDS-PAGE. After staining with Coomassie blue, a band of the expected size for the P1 protein (31 kDa) was clearly visible in the gel. This band was not observed in samples from the non-induced controls (data not shown).

For large-scale production, protein expressed in E. coli was purified as inclusion bodies. The collected cells were lysed with lysozyme (100 µg/ml in 50 mM Tris, pH 8.0, 2 mM EDTA) at 30 °C for 15 min. After lysing, the suspension was sonicated to shear the DNA. Inclusion bodies were collected by centrifugation at 10000 g for 10 min at 4 °C. The pelleted inclusion
Fig. 1. Western blot analysis of antiserum raised against PVY-O P1 protein. Lanes: A, pET3a transformed BL21(DE3)pLysE cells; B, pET3a/P1 transformed, induced BL21(DE3)pLysE cells; C, P1 protein purified as inclusion bodies. Ten µl from each sample (an aliquot equivalent to 100 µl of bacterial culture) was loaded on the gel. The position of P1 protein is indicated to the right and the positions of marker proteins (kDa) to the left.

bodies were washed several times in 50 mM Tris, pH 8·0, 2 mM EDTA. After washing and solubilization of inclusion bodies, the inclusion body protein was purified by preparative electrophoresis on a 10% SDS–polyacrylamide gel (Laemmli, 1970). The bands were visualized by soaking the gel in ice-cold 1 M KCl for 1 min.

A gel slice containing approximately 500 µg of the expressed protein was homogenized in Freund’s incomplete adjuvant (Difco) and injected subcutaneously into a rabbit. Injections were repeated three times at 14 day intervals; 10 ml of blood was collected 14 days after the last injection, and the bleeding was repeated several times at 10 day intervals. Four rabbits were immunized.

The antiserum against P1 protein was tested by Western blotting. Aliquots from non-induced and induced E. coli cultures and the purified P1 protein in 2 × sample buffer (100 mM Tris–HCl, pH 6·8; 4% SDS; 20% glycerol; 0·2% bromphenol blue; 200 mM dithiothreitol) were boiled for 5 min and analysed by SDS–PAGE on a 10% gel. Electrophoresis was done as described by Laemmli (1970). After electrophoresis, proteins were transferred to PVDF membrane (Immobilon-P from Millipore) at 30 V overnight using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were incubated with the P1 antiserum diluted 1:1000 in TBST (20 mM Tris–HCl, pH 7·5; 150 mM NaCl, 0·05% Tween 20). Goat anti-rabbit IgG–alkaline phosphatase conjugate diluted 1:3000 in TBST was used as the secondary antibody. Detection of immunocomplexes was carried out using the ProtoBlot Western Blot AP System (Promega) according to the manufacturer’s instructions. In the induced E. coli, and in the purified P1 protein samples, P1 antiserum reacted with a protein of the expected size, 31 kDa (Fig. 1).

The antiserum produced against P1 protein was used for detection of the P1 protein in infected leaf material. Total protein samples were prepared from 500 mg of leaf tissue from PVY-O-infected (7 days post-inoculation) or healthy tobacco (Nicotiana tabacum). The samples were ground in liquid nitrogen and homogenized in 1 ml of ES buffer (75 mM Tris–HCl, pH 6·1, 4·5% SDS, 9 M urea, 7·5% β-mercapto ethanol, 5 mM PMSF). An equal amount of 2 × sample 

Fig. 2. Detection by Western immunoblotting of the P1 protein in the extracts from PVY-O infected N. tabacum leaf tissue, 7 days post-inoculation. Lanes: A, 10 µl sample of extract from healthy tobacco plant; B, 10 µl sample of extract from PVY-O-inoculated tobacco plants. The position of the P1 protein is indicated to the right and the positions of marker proteins (kDa) to the left.
buffer was added to the plant samples, which were then boiled for 5 min; 10 μl from each protein sample was analysed by SDS–PAGE on a 10% gel. After electrophoresis, proteins were transferred to Immobilon-P membrane, and the Western blot detection of the P1 protein was done as described above. In the Western blots, a protein with a relative molecular mass of 31 kDa was detected in the samples prepared from infected plants, but not in the samples prepared from healthy plants (Fig. 2), suggesting that this virus-specific protein was P1. The P1 antiserum also cross-reacted with plant proteins on Western blotting membranes (Fig. 2), and in an attempt to reduce this background we preadsorbed P1 antiserum with acetone powder of healthy tobacco tissue. However, preadsorption reduced not only the background but also lowered the intensity of the specific signal with P1 protein, suggesting that the P1 antibodies recognize epitopes shared by the PVY P1 protein and some tobacco plant proteins. Western blotting showed that in the sample from infected tobacco leaves two very weak protein bands of lower molecular mass are present; these could be products of proteolysis of the P1 protein (Fig. 2, lane B).

Next, we used the antiserum raised against the P1 protein in conjunction with the immunogold labelling technique. For immunogold labelling, PVY-O-infected (7 days post-inoculation) and comparable healthy N. tabacum plants were placed in the dark overnight prior to tissue processing, to reduce the number of starch grains found in chloroplasts. Leaf strips of about 0·5 × 0·5 mm from systemically infected or healthy leaves were fixed overnight in 2% (w/v) paraformaldehyde and 3% glutaraldehyde in phosphate–citrate buffer, pH 7·2, at 4 °C. Dehydration and embedding of plant tissue in LR Gold resin at low temperature were performed according to van Lent et al. (1990). Thin sections of the plant tissue were harvested on Formvar-coated Ni-grids and treated for 30 min on drops of 1% BSA in TBS (Tris 20 mM, NaCl 500 mM, pH 7·5) to block nonspecific binding of antibodies. The primary P1 antibodies were concentrated by precipitation with ammonium sulfate according to a published protocol (Cooper & Paterson, 1993), and diluted with TBS to a concentration of 50 μg/ml. Sections were incubated with the primary antibodies for 2 h at room temperature or overnight at 4 °C in a humid chamber. After incubation with primary antibodies, sections were washed 3 × 10 min with TBS, incubated for 1·5 h at room temperature with 10 nm protein A–gold (pAg) (Zymed) diluted 1:30 with 50 mM Tris, pH 7·4, 150 mM NaCl containing 1% BSA. Then sections were washed 6 × 5 min with TBS and 2 × 5 min with distilled water before air drying. After immunogold labelling, thin sections were stained with uranyl acetate and alkaline lead citrate and examined with a JEOL JEM-1200EX transmission electron microscope.

Although the P1 antiserum gave a cross-reaction with some plant proteins on Western blotting membranes, no gold label was found in thin sections from non-infected plants incubated with P1 antibodies (Fig. 3a). Most probably, the plant protein epitopes which cross-reacted with the P1 antiserum on Western blotting membranes were masked or modified during fixing and embedding in LR Gold resin. Likewise, no gold label was found on thin sections from infected plants incubated with preimmune serum in spite of the presence of cytoplasmic inclusions within the cells (Fig. 3b).

The occurrence of inclusions in potyvirus-infected cells makes it easy to determine with the electron microscope which cells are infected. PVY encodes a cylindrical inclusion protein that aggregates in the cytoplasm of infected cells to form inclusions resembling pinwheels (Fig. 3b–d) or bundles when seen in longitudinal section (Fig. 3e). Immunogold labelling with P1 polyclonal antibodies localized the P1 protein both in association with cytoplasmic inclusion bodies and in the cytoplasm of infected plant cells of tobacco leaves (Fig. 3c–f). Plasmodesmata were observed in some sections, but no gold label was associated with them (data not shown).

There are several possible explanations why P1 protein is colocalized with cytoplasmic inclusion bodies. A first possibility is that P1 protein localized in association with inclusion bodies because it was synthesized there. It has been shown previously that the coat protein and P3 protein of tobacco vein mottling potyvirus (TVMV) are associated with cytoplasmic inclusions in infected tobacco leaves and proplastids (Ammar et al., 1993, 1994; Rodriguez-Cerezo et al., 1993). Ammar et al. (1994) suggested that synthesis of all the potyviral proteins might occur in, on or near these inclusions, which are usually associated with the rough endoplasmic reticulum.

However, there are other possibilities. With the demonstration of the helicase activity of the cylindrical inclusion protein of a potyvirus (Lain et al., 1990), the replication of potyviral RNA is also likely to be connected with cytoplasmic inclusions. The role of P1 protein in virus infectivity beyond its autoproteolytic activity is still not determined completely. Using tobacco etch potyvirus (TEV) mutants lacking the entire P1 coding region Verchot & Carrington (1995a, 1995b) demonstrated that P1 protein activity is not essential for virus infectivity and that the P1 protein may contribute to but is not strictly required for the TEV RNA amplification. Their data suggest that the P1 protein stimulates viral genome amplification and does not affect cell-to-cell movement. If cytoplasmic inclusions are indeed sites of potyviral RNA replication, the present results on the localization of the P1 protein on the cytoplasmic inclusion bodies are consistent with the hypothesis of Verchot & Carrington that P1 protein could participate in virus replication. However, further experiments will be necessary to determine the role of P1 protein in the virus infection cycle more precisely. Finally, the absence of the PVY P1 protein from the cell wall and plasmodesmata is also consistent with the results of Verchot & Carrington on the P1 protein of TEV, suggesting that P1 is not involved in virus or
Fig. 3. Immunolocalization of the P1 protein in thin sections of cells from systemically infected *N. tabacum* leaf tissue, 7 days post-inoculation with PVY-O. (a) Section from healthy tobacco leaf treated with antibodies to PVY P1 protein. (b) Section from infected tobacco leaf incubated with preimmune rabbit serum. (c–f) Thin sections of infected cells incubated with antibodies to PVY P1 protein. CI, cytoplasmic inclusions; Ch, chloroplast; CW, cell wall; M, mitochondrion; Mb, microbody; N, nucleus; Nu, nucleolus; S, starch grain; V, vacuole. Bar, 200 nm.
viral RNA movement through the plasmodesmata of PVY-infected cells.

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References


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