‘Pathogenesis-related’ P1(p14) Protein. Vacuolar and Apoplastic Localization in Leaf Tissue from Tomato Plants Infected with Citrus Exocortis Viroid; in vitro Synthesis and Processing

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SUMMARY

Tomato ‘pathogenesis-related’ P1(p14) protein was synthesized in vitro. mRNAs were isolated from leaves showing characteristic symptoms of viroid infection, followed by chromatography on oligo(dT)-cellulose and the poly(A) + fraction was translated with a rabbit reticulocyte lysate system. No significant differences were found between the levels of [35S]methionine incorporation directed by mRNA preparations from healthy and viroid-infected leaves. Only mRNA from infected leaves incorporated label into a protein that could be immunoprecipitated with rabbit IgG specific for tomato P1(p14) protein. Analysis by SDS-PAGE of the immunoprecipitated protein from the in vitro translation system revealed that P1(p14) was translated as a precursor protein of 2K to 3K larger than the P1(p14) that accumulated in vivo. This protein was converted to the mature form when translation was carried out in the presence of canine pancreatic microsomal membranes. By using Protein A–gold immunocytochemistry we have detected P1(p14) concentrated in dense inclusion bodies within the vacuole as well as in association with electron-dense material present in the intracellular spaces. The presence of the additional polypeptide sequence in the newly synthesized protein indicates that P1(p14) undergoes post-translational processing. The additional sequence is probably the signal peptide that directs its transport through the endoplasmic reticulum into the vacuolar compartment of tomato leaf cells and/or the intercellular space.

INTRODUCTION

Infection of plants with pathogens results in the induction and accumulation of a family of proteins named pathogenesis-related (PR) proteins. The production of these proteins may be part of a general mechanism of response, programmed by the plant genome, which is activated after pathogen attack or after treatment with different stress-causing agents (van Loon, 1985). Recent studies have shown that some PR proteins are hydrolytic enzymes: β-1,3-glucanases (Kauffmann et al., 1987; Kombrink et al., 1988), chitinases (Kombrink et al., 1988; Legrand et al., 1987; Nasser et al., 1988) and a protease (Vera & Conejero, 1988a, b). These enzymes are considered to play an important role in defence reactions.

Tomato plants respond to citrus exocortis viroid (CEV) infection with the synthesis and accumulation of a set of basic PR proteins (Granell et al., 1987; Vera et al., 1988; Vera & Conejero, 1988a, b). One of these proteins, P1(p14), has also been connected with natural senescence in healthy plants (Camacho Henriquez & Singer, 1982). Consistent with this observation, P1(p14) has recently been located in association with disorganized cytosol of certain decaying cells and in the intercellular spaces (or apoplasts) of healthy (non-infected) leaf tissue. Consequently, the idea that P1(p14) is involved in cell degeneration has been proposed (Vera et al., 1988).
This paper reports the immunocytochemical localization of P1(p14) in CEV-infected tomato leaf tissue by using the same immunocytochemical approach described previously (Protein A-gold electron microscopy) (Vera et al., 1988). We show that P1(p14) protein is located in the apoplast and in the central cell vacuole. We have also determined that P1(p14) is synthesized in the form of a precursor protein (pre) as observed for other plant proteins which also accumulate in vacuoles (Nelson & Ryan, 1980; Broglie et al., 1986; Cleveland et al., 1987). In addition, we show that preP1(p14) can be processed to its mature form when canine microsomal membranes are present during translation.

**METHODS**

**Plant material.** Tomato plants (*Lycopersicon esculentum* Mill, CV. Rutgers) were grown from seeds in a greenhouse at 25 to 30 °C. Inoculation with a purified preparation of CEV was performed at an early stage previously (Granell et al., 1987).

**Isolation of P1(p14) and preparation of antiserum.** PR protein P1(p14) was purified from viroid-infected tomato plants using a procedure described elsewhere (Vera et al., 1988). An antiserum was produced in rabbits and its specificity has already been described (Vera et al., 1988).

**Electrophoresis and fluorography.** Electrophoresis was performed in 14% polyacrylamide gels containing SDS as described (Conejero & Semancik, 1977). The gels were then impregnated with Amplify (Amersham), dried, and then exposed to Kodak XAR5 film. [35S]Methionine was quantified by liquid scintillation.

**Determination of P1(p14) density.** Electron micrographs of sections were taken and printed at a magnification of ×40000. P1(p14)-associated gold particles were counted and attributed to each cell compartment. A transparent sheet with a grid (1cm-spaced parallel lines) was superimposed on the prints and the number of gold particles per unit area was determined. The proportion of gold particles in each cell compartment was used as a measure of P1(p14) density.

**Immunocytochemistry.** Leaves showing characteristic symptoms of viroid infection were collected 20 days after inoculation. The leaves were cut under water into 2 × 1 mm strips and fixed with 1% formaldehyde-0.7% glutaraldehyde in potassium phosphate buffer, 1 mM-CaCl2 (pH 7.2) for 2 h at 4 °C as previously described (Vera et al., 1988). Tissue blocks were dehydrated, infiltrated with Lowicryl K4M resin and polymerized under u.v. light as described (Vera et al., 1988). Thin sections were cut and mounted on 200-mesh nickel grids covered with carbon film and floated on 1% ovalbumin in phosphate-buffered saline (PBS) pH 7.2 for 30 min at 4 °C. The sections were transferred to P1(p14) antiserum diluted 100-fold in PBS-ovalbumin and processed for Protein A-gold labelling as previously described (Vera et al., 1988). The sections were stained in the dark for 20 min with 5% aqueous uranyl acetate and for 45 s with alkaline lead citrate.

**Extraction of poly(A)+ mRNA.** Total RNA was extracted from leaf tissues according to a procedure derived from Chirgwin et al. (1979). Tomato leaves (25 g) from either healthy or CEV-infected plants were frozen in liquid nitrogen and ground in a mortar. The powder was mixed rapidly with 10 ml of 50 mM-Tris-HCl pH 8.0, containing 10 mM-EDTA, 5 mM-guanidinium thiocyanate, 2% sodium N-lauroylsarcosine and 5% v/v 2-mercaptoethanol. After incubation at 60 °C for 10 min and centrifugation at 20000 g for 30 min, the supernatants were adjusted to 0.1 g/ml CsCl and layered on 12 ml cushions of 5.7 M-CsCl, 50 mM-EDTA, pH 8.0, in 30 ml polyallomer tubes. The tubes were centrifuged for 24 h at 25000 r.p.m. in an SW27 rotor at 18 °C. The RNA pellets were then dissolved in a small volume of 25 mM-sodium citrate, 7.5 mM-guanidine hydrochloride, 5 mM-dithiothreitol and RNA was precipitated by adding 0.025 volumes of 1 M-acetic acid and 2.5 volumes of ethanol. The final pellets were dispersed in ethanol and centrifuged for 5 min at 6000 r.p.m. The firm pellets were vacuum-dried and the RNA was dissolved in 1 ml sterile distilled water and precipitated by adding 0.1 volumes of 2 M-potassium acetate, pH 5.0 and 2.5 volumes of ethanol. This was left overnight at −20 °C. The yield was generally in the range of 200 to 300 μg total RNA per g of fresh tissue. Poly(A)+ mRNA was purified by two cycles of selection on an oligo(dT)-cellulose column (Sigma) according to the procedure of Maniatis et al. (1982).

**Cell-free translation of mRNAs, processing and immunoselection of the translation products.** Total poly(A)+ mRNAs were translated in a rabbit reticulocyte cell-free translation system (Amersham) using [35S]methionine (900 Ci/mmol, 1 mCi/ml) (Amersham). Approximately 0.1 μg poly(A)+ mRNA and 1 μCi [35S]methionine were added per μl of lysate. After incubation for 1 h at 35 °C, aliquots of the translation mixture were analysed by SDS-PAGE. Cell-free translation products were processed by the addition of canine pancreatic microsomes (New England Nuclear), at the indicated concentration, to the reaction mixture containing the reticulocyte lysate system. Translation products corresponding to 50 μl of lysate were immunoselected for P1(p14) as follows. The
translation products were diluted by the addition of 100 μl of buffer A (10 mM-Tris–HCl pH 7.3, 150 mM-NaCl, 10 mM-EDTA, 1% Triton X-100). The samples were centrifuged at 100000 g for 45 min and 15 μl of anti-P1(p14) serum was added to the supernatants. Following incubation for 12 h at 4°C, 100 μl of Protein A–Sepharose CL-4B (Pharmacia) (10 μg/ml) prepared in buffer A was added and incubation was continued for 1 h at 20°C, after which the Protein A–Sepharose with the attached antigen–antibody complexes were sedimented by centrifugation through a cushion of 1 M-sucrose prepared in buffer A, followed by three washes in buffer A. The pellets were analysed by SDS–PAGE.

In vivo labelling of P1(p14). In vivo labelling was performed by incubation of CEV-infected tomato leaf discs (0.5 cm in diameter) with [35S]methionine (1000 Ci/mmol, 200 μCi/ml; in vivo labelling grade, Amersham) for 3 h. After labelling the discs were homogenized in 84 mM-citric acid–32 mM-NaHPO4 pH 2.8, containing 15 mM-2-mercaptoethanol. The homogenates were centrifuged for 20 min at 20000 g and the supernatants were adjusted to pH 7.3 with 1 M-Tris-HCl. Radiolabelled P1(p14) was immunoprecipitated with anti-P1(p14) serum as described above.

RESULTS

Intracellular localization of the PR protein P1(p14)

Immunocytochemical localization showed that P1(p14) is located in dense inclusion bodies present in the cell vacuole (Fig. 1 a to f). Control sections treated with preimmune serum (Fig. 2e and Fig. 3), Protein A–gold alone or anti-P1(p14) antiserum saturated with a large excess of P1(p14) protein (not shown) had no specific labelling in the vacuolar inclusion bodies. No significant labelling of the cytoplasm was found for P1(p14) (see Table 1 and compare the gold particle distribution on nuclei, mitochondria, chloroplasts, microbodies and cell walls).

The distribution of gold label within vacuolar inclusion bodies was prominent and no significant variations could be found between cells. These inclusion bodies were found to be present in 30 to 40% of the cells analysed on every section of parenchyma leaf tissue from CEV-infected plants. We have not found these inclusion bodies in leaf tissue from healthy (non-infected) plants. No traces of P1(p14) as a free protein in vacuoles could be found (data not shown). In healthy plants, P1(p14) protein has been detected in association with only a disorganized cytosol found in a few cells of the parenchyma tissue and also in some intercellular spaces of mature tomato leaves (Vera et al., 1988).

Intercellular localization of P1(p14) protein

Immunogold labelling of CEV-infected leaf sections revealed that P1(p14) was also present in intercellular spaces, and always in association with electron-dense material. This material has also been found associated with P1(p14) protein in preparations from healthy (non-infected) tomato leaf tissue (Vera et al., 1988). It must be emphasized that in CEV-infected preparations, the abundance of both electron-dense material and the anti-P1(p14) gold label in intercellular spaces was dramatically enhanced with respect to healthy preparations [see Table 1 and Fig. 2, and compare with those previously published (Vera et al., 1988)]. Control sections treated with preimmune serum (Fig. 2e, 3a to c and Table 1), presaturated antiserum or Protein A–gold alone (not shown) showed no specific labelling in these compartments.

In vitro synthesis of tomato PR protein P1(p14)

RNA was extracted from healthy and CEV-infected tomato leaves 20 days after inoculation with CEV. At this early growth stage, leaf tissue from healthy plants had no detectable P1(p14) content. This protein however, is normally found in older leaves (Camacho Henriquez & Singer, 1982; Vera et al., 1988). One percent to 1·3% of the total RNA in either healthy or CEV-infected leaves was present as poly(A)+ mRNA. Maximum incorporation of radioactivity from [35S]methionine was achieved with 3 to 4 μg poly(A)+ mRNA per translation assay. For analysis of immunoprecipitable material, 50 μl of translation mixture was used. About 0·12% of radioactivity incorporated into protein was recovered in anti-P1(p14) immunoprecipitation assays containing poly(A)+ mRNA from CEV-infected tomato leaves; negligible radioactivity was obtained with poly(A)+ mRNA from healthy leaves.

Further analysis by SDS–PAGE and fluorography revealed that the immunoprecipitable material from the translation assays was concentrated in a single band of Mr 16000 to 16500.
Fig. 2. (a to e) Immunocytochemical localization of P1(p14) on thin sections of tomato mesophyll cells. Sections were incubated with P1(p14) antiserum (a to d) or with preimmune control serum (e), followed by incubation with Protein A–colloidal gold as described in Methods. Key, as described for Fig. 1. Bar markers represent 1 µm.

Fig. 1. (a to f) Immunocytochemical localization of P1(p14) in vacuolar inclusion bodies of palisade parenchyma cells from CEV-infected tomato leaves. Vacuolar inclusion bodies (denoted by arrows in a) in different cells differ in size, shape and complexity (a to f). P1(p14) is indicated by the gold particles concentrated over the inclusion bodies (IB). M, mitochondrion; V, vacuole; CHL, chloroplast; IS, intercellular space. Bar markers represent 1 µm.
Fig. 3. (a to c) Control sections incubated with preimmune serum and Protein A–colloidal gold, showing essentially no staining either in vacuolar inclusion bodies or in intercellular spaces. Key as described for Fig. 1. Bar markers represent 1 μm.

Table 1. Density of gold labelling on palisade parenchyma cells of CEV-infected tomato leaves using anti-P1(p14) and control sera

<table>
<thead>
<tr>
<th>Cell compartment</th>
<th>Anti-P1(p14)</th>
<th>Control serum</th>
<th>Corrected density†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercellular space</td>
<td>140 ± 12</td>
<td>3.2 ± 0.6</td>
<td>137 ± 11</td>
</tr>
<tr>
<td>Cell wall</td>
<td>2.5 ± 0.6</td>
<td>3.4 ± 1.3</td>
<td>–</td>
</tr>
<tr>
<td>Nucleus</td>
<td>11 ± 3.1</td>
<td>8 ± 0.7</td>
<td>–</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>14 ± 2.4</td>
<td>9 ± 3.2</td>
<td>–</td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>1.2 ± 0.3</td>
<td>4.5 ± 2.0</td>
<td>–</td>
</tr>
<tr>
<td>Vacuole (inclusion body)</td>
<td>127 ± 8.4</td>
<td>6.3 ± 3.3</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>Ground cytosol</td>
<td>7.4 ± 3.5</td>
<td>6.2 ± 2.7</td>
<td>–</td>
</tr>
</tbody>
</table>

* For each antiserum, gold labelling was examined in each cell compartment in a number of samples accounting for a total surface area exceeding 140 μm². Values are means (n = 3) ± S.D.
† Corrected value is the labelling density for anti-P1(p14) minus the labelling density for control antiserum.

(Fig. 4). As immunoprecipitation of translation products in the presence of 3 μg of unlabelled P1(p14) (Fig. 4, lane 6) or with preimmune serum (lane 8) produced no radioactive band, we conclude that the band represents a precursor protein larger than P1(p14), the mature form of which has an Mr of approximately 14000 (Fig. 4, lane 4) as determined after immunoprecipitation and SDS–PAGE of P1(p14) synthesized in vivo.

The mRNAs isolated from leaves of CEV-infected and those from healthy plants were virtually identical in their capabilities to direct the incorporation of [35S]methionine into the bulk of proteins. However, only the mRNA isolated from leaves of CEV-infected plants directed the incorporation of [35S]methionine into immunoprecipitable P1(p14) in the in vitro translation system (Fig. 4, lanes 5 and 7). This suggests that CEV infection, as well as other stress-causing agents and even senescence, induces synthesis of new mRNAs. This is in agreement with the discovery that some tobacco and parsley PR proteins synthesized on membrane-bound polysomes are regulated at the level of transcription (Carr et al., 1985; Somssich et al., 1986; Hooft van Huijsduijnen et al., 1985).
Fig. 4. SDS-PAGE and fluorography of proteins synthesized in vitro and in vivo. Lanes 1 and 2 correspond to the in vitro synthesized proteins in reticulocyte lysates programmed with poly(A)+ mRNA from healthy and CEV-infected plants respectively. Lanes 3, 4, 5 and 6 represent antigen–antibody competition experiments to determine the relationship of in vivo (lanes 3 and 4) and in vitro (lanes 5 and 6) [programmed with poly(A)+ RNAs from CEV-infected plants] synthesized P1(p14). Immunoprecipitation of P1(p14) was performed in the presence (lanes 3 and 6) or absence (lanes 4 and 5) of 3 μg of purified P1(p14). Lane 7 represents immunoprecipitation with anti-P1(p14) serum of in vitro translation products when poly(A)+ RNAs from healthy plants were used to programme the reticulocyte synthesis reaction. Lane 8 represents immunoprecipitation with preimmune serum of in vitro translation products when poly(A)+ RNAs from CEV-infected plants are used to programme the synthesis.

Processing of preP1(p14) by microsomal membranes

The P1(p14) protein detected by the immunological method and synthesized in the reticulocyte system in the presence or absence of canine microsome membranes is shown in Fig. 5. The results clearly demonstrate that the precursor protein was processed to the mature form when microsomes were present during translation. This result strongly suggests that the P1(p14) protein has a signal peptide which is removed during its translocation into the lumen of the endoplasmic reticulum.

DISCUSSION

Using Protein A–gold immunocytochemistry and an antibody against tomato leaf protein P1(p14) we have found that the latter accumulates in two different locations of the CEV-infected leaf tissue: in intercellular spaces, as observed for other PR proteins (Parent & Asselin, 1984; Carr et al., 1987; Dumas et al., 1988; Hosokawa & Ohashi, 1988) and, without precedent, in the cell vacuole.

The clear-cut localization of P1(p14) within vacuoles, and always in association with inclusion bodies, introduces further aspects on the interpretation of the physiological role of PR proteins. The possibility that inclusion bodies found in vacuoles are artefacts of tissue preparation can be excluded because they have not been detected in similarly prepared leaf tissue sections from healthy plants (data not shown; see Vera et al., 1988). The nature of these inclusion bodies is as yet unknown. They have also been found in tobacco cell vacuoles during adaptation to high osmotic stress, associated with ‘osmotin’, a protein which is highly homologous to a tobacco mosaic virus-induced PR protein in tobacco plants (Singh et al., 1987). By analogy, it can be said that vacuolar inclusion bodies may function within vacuoles as a type of storage compartment
for PR proteins, particularly P1(p14). In fact, none of these vacuolar inclusion bodies has been found without P1(p14). This localization has also been suggested for the salt-induced NP-24 protein of tomato plants (King et al., 1988) and for the sweet-tasting protein 'thaumatin' (Edens & van der Wel, 1985), both highly homologous to the tobacco protein osmotin. In addition, P1(p14)-like PR proteins have been found in several plant species under stress from different agents (White et al., 1987). Taking all this into account the possibility exists that vacuolar inclusion bodies are a general feature of the reaction of plants to biological or non-biological afflicting agents.

The fact that P1(p14) is synthesized as a higher Mr precursor is in agreement with the idea that this protein must be transported within the cell. It implies that P1(p14) undergoes post-translational modification to generate the shorter mature protein found to accumulate in vivo. This argument is validated by the finding that microsomal membranes are able to convert preP1(p14) to its mature form, thus being consistent with the observation on other vacuolar proteins which lose an N-terminal domain on their journey to the vacuole [e.g. tomato and potato proteinase inhibitors (Nelson & Ryan, 1980; Cleveland et al., 1987; Graham et al., 1985) or chitinase (Broglie et al., 1986; Boller & Vogeli, 1984)], and also with the fact that a P1(p14)-like PR protein of the tobacco plant is derived from a precursor by the removal of an N-terminal signal peptide of 30 amino acids (Hooft van Huijsduijnen et al., 1985). The mechanism accounting for the localization of P1(p14) in both vacuoles and intercellular spaces is still a matter of conjecture. One possible explanation, as suggested for bean chitinase (Boller & Vogeli, 1984), is the occurrence of vacuoles breaking and discharging their contents into intercellular spaces after dissolution of the cell (e.g. in a hypersensitive reaction) as part of the pathogenetic process. Also, the possibility that once in the endoplasmic reticulum, P1(p14) can be sorted via alternative pathways either to the apoplast or to the vacuole cannot be disregarded. The elucidation of these questions may be fundamental to our understanding of the mechanism of compartmentation of PR proteins and of their biological role.

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**PR protein in viroid-infected tomato plants**

### REFERENCES


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