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Immunogold Localization of the Citrus Exocortis Viroid-Induced Pathogenesis-Related Proteinase P69 in Tomato Leaves[1]

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ABSTRACT

Citrus exocortis viroid induces in tomato plants (*Lycopersicon esculentum*) synthesis and accumulation of a pathogenesis-related protein (P69) previously reported to be a proteinase (Vera P, Conejero V [1988] Plant Physiol 87: 58-63). By immunogold/transmission electron microscopy, we have studied the distribution of this protein in thin sections of parenchymatous leaf tissue. The enzyme was present intra- and extracellularly. The intracellular location was limited to the vacuole and was always associated with engulfed cell material. When extracellularly located, the enzyme was associated with a dispersed, electron-dense material in the intercellular spaces. This latter location was confirmed after analysis of intercellular washing fluids obtained by vacuum infiltration of leaves. These observations provide new data for the understanding of viroid pathogenesis and the biological role of the pathogenesis-related proteinase P69.

Since the discovery of the PR[2] proteins in tobacco (5, 23), it has been demonstrated that this response occurs in a great variety of plants. That is, pathogenic attack induces a small number of specific genes to produce mRNAs to permit synthesis of a similar number of specific proteins. Furthermore, synthesis of some or all of these proteins is induced by many other forms of stress. As reviewed by Van Loon (22), it appears that one or more of the proteins may have a physiological role in resistance to these stressful situations.

As a consequence of viroid infection, tomato plants respond with the development of a pathological syndrome that is accompanied by *de novo* synthesis of several PR proteins (6). In this biological system, one of the newly synthesized PR proteins is an alkaline proteinase, that we have named proteinase P69 (24). This protein, as with other PR proteins, is also induced by ethylene (25). Ethylene may be acting as a mediator in the plant response to viroid infection as in other plant-pathogens interactions (6, 16).

Other PR proteins are endowed with 1,3-β-glucanase and chitinase activities (7–9, 12 ,13). These properties suggest that plants possess a large arsenal of lytic enzymes inducible in stress responses. These enzymes may participate in biochemical defenses against pathogens.

Here we present results of an immunological approach to determine the cellular site of accumulation of proteinase P69 in tomato leaf tissues infected with CEV. We have used a monospecific antiserum against P69 proteinase (25) to localize this protein by immunogold staining. We have found P69 in the vacuole and intercellular spaces of leaf parenchyma cells. In the vacuole, P69 appears specifically associated with defined electron dense material, which is similar to the "inclusion bodies" previously described in tobacco cell vacuoles after saline stress (18), and in tomato leaves after physical injury (17). The intercellular space localization was confirmed by the recovery of P69 in IWF after vacuum infiltration of leaf tissue. In view of this dual location of P69, we have also considered a mechanism that might explain the possible biological roles of the viroid-induced proteinase P69.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum*) were grown from seeds in a greenhouse at 25 to 30°C. Inoculation with purified preparations of CEV was performed at an early stage of growth as previously described (24).

Isolation of CEV-Induced P69 Proteinase and Preparation of Antisera

PR proteinase P69 was purified from CEV-infected tomato plants by affinity chromatography on casein-Sepharose as previously described (24). Antisera were produced in New Zealand rabbits, and their specificity has been described elsewhere (25).

Electrophoresis, Zymography, and Immunoblotting

Electrophoresis and immunoblotting were performed as described elsewhere (24, 26). A 1:200 dilution of anti-P69 antiserum was used (25). The immobilized conjugates were detected with horseradish peroxidase-conjugated goat-anti-rabbit IgG (Bio-Rad) and color development was achieved with 4-chloro-1-naphthol (Sigma) as substrate. Zymography

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[2] Abbreviations: PR, pathogenesis-related; CEV, citrus exocortis viroid; IWF, intercellular washing fluids; FTC, fluorescein thiocarbamoyl derivative.

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was performed in 14% SDS-gels containing 2 mg/ml of fibrinogen (Sigma) as described (25).

Quantification of Tomato P69 Protein Content

P69 protein was quantified by immunoradial diffusion (25), with purified tomato proteinase P69 as a standard. Proteinase activity was measured with FTC-casein as substrate (24).

Extraction of Intercellular Fluids from Tomato Leaves

IWF from either CEV-infected or healthy tomato leaves were obtained by cutting leaf pieces (2 x 2 cm) in water and blotting dry on Whatman 3MM paper. The leaf pieces were infiltrated *in vacuo* with gentle shaking, either in distilled water or in the presence of different salts and buffers for 30 s at prefixed pressures by means of a calibrated vacuum pump

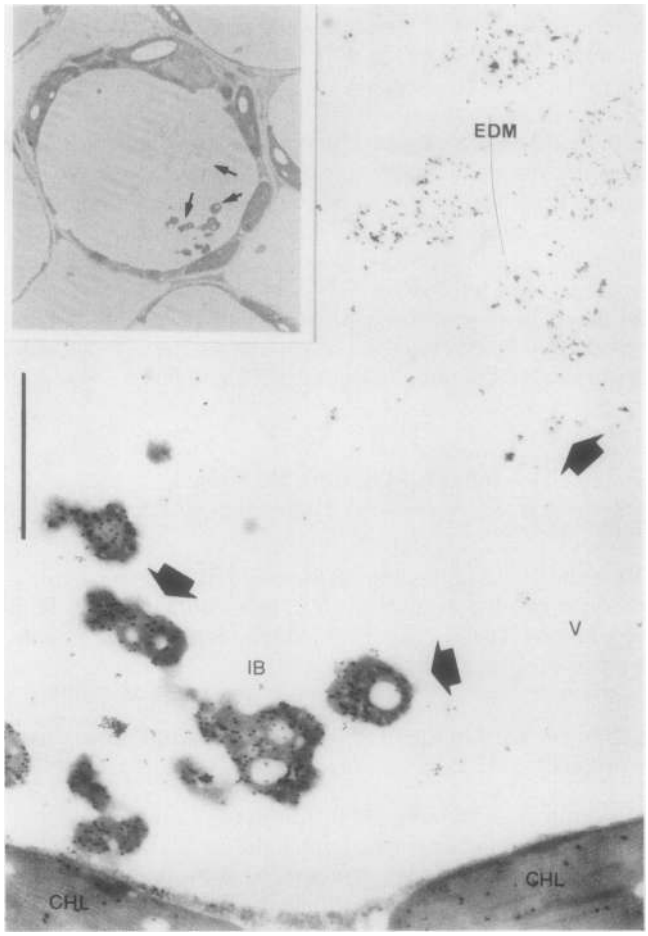

Figure 1. Immunocytochemical localization of proteinase P69 in CEV-infected tomato leaves. The primary antiserum has labeled vacuolar inclusion bodies (*IB*) and electron-dense material (*EDM*) clearly defined in the central cell vacuole. Different organelles and the remainder of the cytoplasm are labeled at the level of background (see Table I and control sections in Fig. 2). The *insert* in the figure corresponds to lower magnification micrographs in order to show the cellular context that surrounds the inclusion bodies (indicated by *arrows*). Key: chloroplast (*CHL*), vacuole (*V*), inclusion body (*IB*), electron-dense material (*EDM*). Bar: 1 μm.

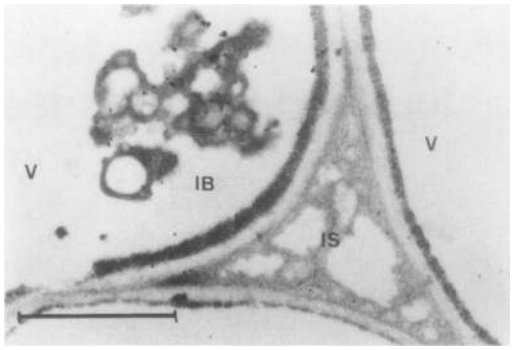

Figure 2. Control sections of parenchymatous cells from CEV-infected leaf tissue. Sections were incubated with preimmune serum prior to incubation with protein A-gold. Abbreviations as described in Figure 1. Bar: 1 μm.

Table I. *Density of Gold Labeling on Palisade Parenchyma Cells of CEV-Infected Tomato Leaves Using Anti-P69 and Control Serum*

For each cell compartment and for each antiserum, the gold labeling was examined in a number of samples accounting for a total surface of at least 90 μm^2 for each organelle. Values are means (n = 3) ± SD.

Cell Compartment	Labeling Density		
	Anti-P69	Control serum	Corrected[a]
	gold particles/μm^2		
Cell Wall	3.7	2.6	
Chloroplast	16.6 ± 3.0	10 ± 2.8	6 ± 3
Ground cytosol	9.8 ± 5.3	7.1 ± 3.6	3 ± 1.5
Intercellular space	125 ± 9.3	4 ± 0.3	120 ± 4
Mitochondrion	2 ± 0.5	3.4 ± 1.1	
Nucleus	14 ± 4.8	7.7 ± 1.8	6 ± 2.5
Vacuole (inclusion body)	116 ± 5.2	5.7 ± 1.9	110 ± 5

[a] Corrected = (labeling density for anti-P69) − (labeling density for control antiserum).

(Millipore) at 4°C. IWF were recovered by centrifugation of the infiltrated leaves at 2000*g* for 5 min in specially prepared centrifuge tubes perforated at the bottom (15). IWF were directly analyzed for P69 content or stored frozen at −70°C.

Immunocytochemical Methods

Leaves were harvested 20 d after inoculation of tomato plants with CEV, when they had expanded, were showing symptoms, and possessed the highest amount of the CEV-induced proteinase P69 (25). Leaves of the same growth stage from noninoculated plants were taken as control. The leaves were cut into 2 x 1 mm strips under water and fixed with 1% formaldehyde-0.7% glutaraldehyde as previously described elsewhere (26). Tissue blocks were dehydrated, infiltrated, and polymerized in Lowicryl K4M resin (Polaron Equipment Limited, Bio-Rad) as described previously (26). Ultrathin sections were cut and mounted on 200-mesh nickel grids, covered with carbon film, and floated for 15 min at 20°C on 1% ovalbumin (Sigma) prepared in PBS (20 mM PBS [pH 7.3], 0.9% [w/v] NaCl). Sections were then transferred to P69 antiserum diluted 1:100 in PBS and processed for protein A-

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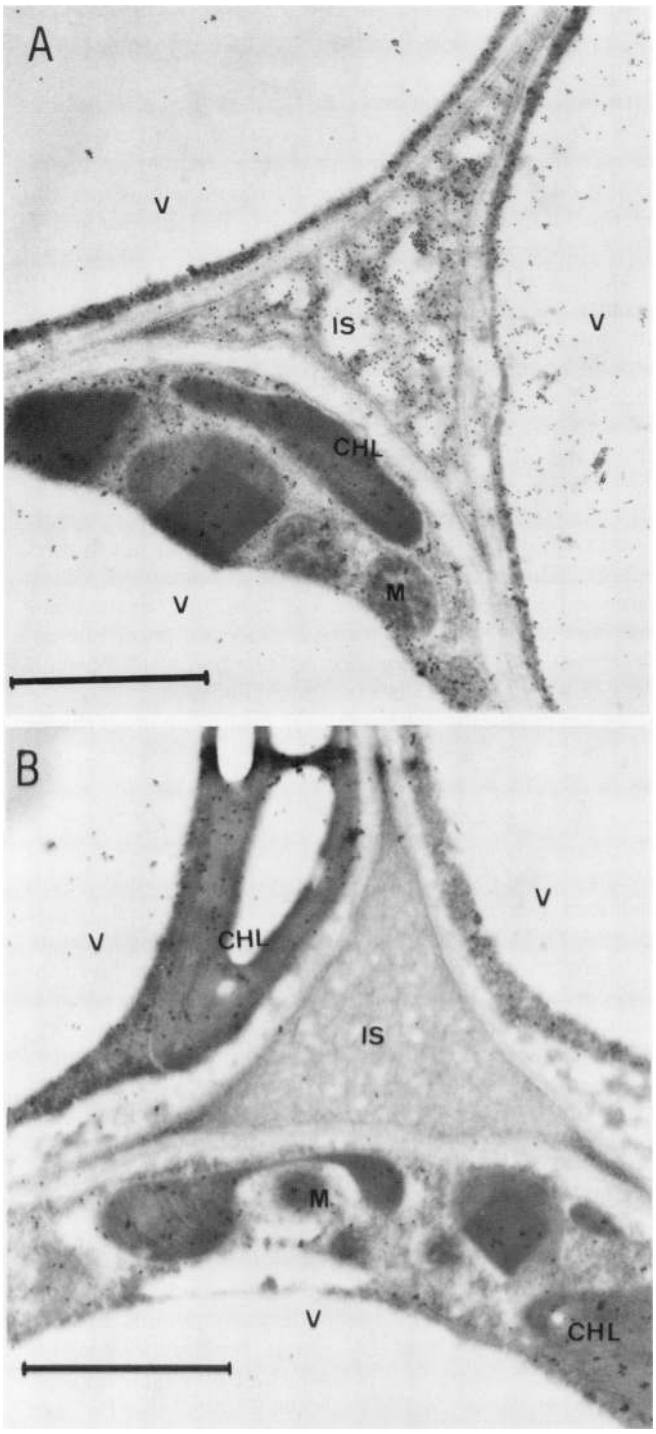

Figure 3. Localization of P69 proteinase in the intercellular spaces of CEV-infected leaf tissue. A, Sections treated with P69 antiserum. B, Sections treated with preimmune serum. Key: intercellular space (*IS*), mitochondrion (*M*), and others as defined in Figure 1. Bar: 1 μm.

gold (freshly prepared, 10–20 nm diameter) labeling as previously described (26). Grids were stained in the dark for 20 min with 5% aqueous uranyl acetate and for 45 s with alkaline lead citrate. Control sections were similarly treated with preimmune serum diluted 1:100 in PBS, protein A-gold alone,

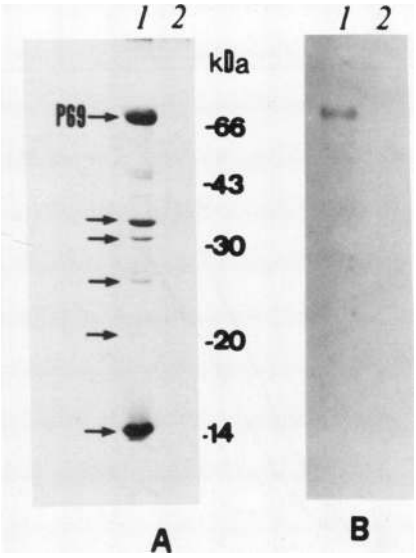

Figure 4. SDS-PAGE and immunoblotting analysis showing the presence of P69 proteinase in the IWF. A, Coomassie blue staining of the proteins recovered in the IWF from CEV-infected (1) and healthy (2) tomato leaves. B, Western blot of samples shown in (A), incubated with P69 antiserum. IWF were obtained after infiltration of 1 g of tomato leaf pieces for 30 s at 64 cm Hg and centrifugation as described in "Materials and Methods." Volumes of 50 μl of IWF were analyzed in each lane.

or with anti-P69 serum absorbed with a large excess of purified P69 proteinase. Electron microscopy was carried out in a Philips 300 microscope at 80 kV.

RESULTS

CEV-infected leaf tissue gold-labeled with anti-P69 antiserum showed a clear-cut localization of proteinase P69 in two different compartments: vacuoles and intercellular spaces.

The gold-labeled P69 was concentrated in the central vacuole associated with dense "inclusion bodies" as well as with dispersed material (Fig. 1). Control sections treated with preimmune serum (Fig. 2) showed no specific labeling in the material contained within vacuoles (Table I). Inclusion bodies were found mainly at the periphery of the vacuole (often in contact with the tonoplast). The disperse electron-dense material, however, occupied a more inner position in the vacuole. Both inclusion bodies and electron-dense material were found to be present in 20 to 30% of cells per section of palisade parenchyma tissue. Size, complexity, morphology, and number of inclusion bodies differed from cell to cell, but the immunogold-labeling density for P69 proteinase was essentially the same (Table I). Vacuolar inclusion bodies were not present in sections prepared from healthy leaf tissue (data not shown). The possibility that inclusion bodies represent cross sections through finger-like cytoplasmic protrusions into the vacuole was ruled out by studying serial sections (data not shown).

In addition to this vacuolar localization, P69 proteinase was found associated with electron-dense material in intercellular spaces (apoplast) of parenchyma (Fig. 3).

The immunogold labeling in the rest of the cell was similar

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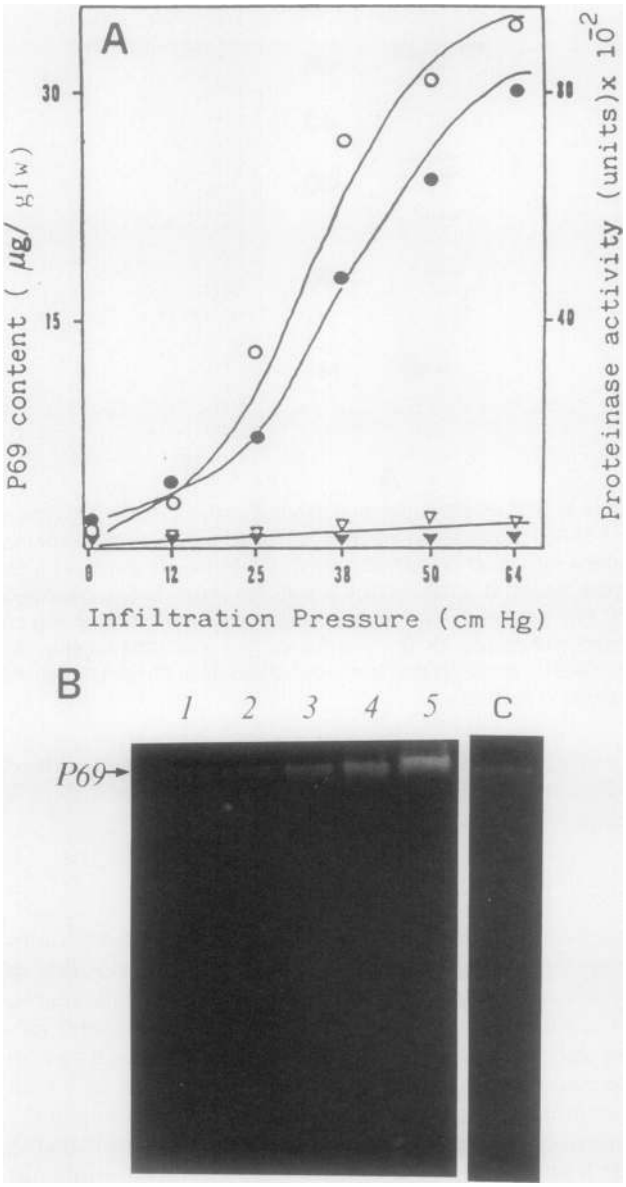

Figure 5. Recovery of P69 proteinase from intercellular spaces of healthy and CEV-infected tomato leaves. IWF were recovered by centrifugation after infiltration of leaf segments for 30 s at the indicated vacuum pressures. A, After concentration by freeze-drying, equivalent amounts of IWF from CEV-infected and healthy plants were assayed for P69 content by radial immunodiffusion (○ = infected, ▽ = healthy) and for P69 proteinase activity in Tris-HCl (pH 9.0), 1 mM DTT, and 1 mM CaCl, with FTC-casein as substrate (● = infected, ▼ = healthy). Values are means of three different extractions. B, Equal volumes (25 µL) of IWF from CEV-infected were assayed for P69 proteinase activity by zymography on fibrinogen-containing SDS-gels after infiltration of leaf segments (1 g) for 30 s with different vacuum pressures. Numbers 1 through 5 correspond to IWF obtained after infiltration at 12, 25, 38, 50, and 64 cm Hg, respectively. C, control experiment with 3 µg of purified P69 proteinase.

to the background observed in the control sections treated with preimmune serum or protein A-gold alone (Table I, Figs. 2, 3B).

An extracellular location of P69 protein was also demonstrated by SDS-PAGE of the IWF. A majority of the proteins corresponded to PR proteins previously reported in tomato (6) (Fig. 4). These proteins included a protein which was determined by immunoblotting to be the P69 proteinase (Fig. 4B). Zymography and hydrolysis of FTC-casein demonstrated the protein in the IWF to possess proteolytic activity (Fig. 5, A and B). Recovery of the protein, as quantified by radial diffusion assay (Fig. 5A), after infiltration with different pressures, increased up to a maximum of 25 to 30 µg in IWF per gram of leaf tissue. By comparing the amount of P69 in IWF and whole tissue extracts (25), it appeared that at least 30 to 40% of the total P69 proteinase was located outside the cell. The addition of different salts (*e.g.*, NaCl, KCl, $MgCl_2$, or $CaCl_2$) to the infiltrating solution did not significantly increase the recovery of P69 from intercellular spaces (data not shown). The possibility that P69 was in IWF as a consequence of cell disruption during manipulation and infiltration of the tissue was ruled out, because no traces of glucose-6-phosphate dehydrogenase, Chl, or immunoreactive ribulose-1,5-bisphosphate carboxylase were detected (data not shown).

P69 was practically undetectable in healthy plants as shown in Figures 4 and 5 (24, 25).

DISCUSSION

We have provided evidence that proteinase P69 is located in vacuoles and in intercellular spaces. In the vacuole, P69 is associated with engulfed 'inclusion bodies' and with dispersed material, which could be an advanced stage of disorganization of the inclusion bodies. In the intercellular spaces, the proteinase was found associated with an electron-dense material resembling the residual stage of cytosol disorganization previously described in the study of the localization of tomato PR P1-p14 protein (26). The extracellular localization of proteinase P69 is, therefore, consistent with the general consideration that PR proteins accumulate in the apoplast (4, 15, 22, 26).

The vacuolar localization of P69 proteinase is consistent with the concept that the central vacuole might be a 'defense arsenal' (1, 10). Other lytic or defense proteins (*e.g.* chitinases [3], proteinase inhibitors [27], and proteinases [14, 19, 29]) as well as many preformed secondary compounds are also compartmentalized within the vacuole. Besides the consideration of P69 proteinase as a potential defense weapon against attacking pathogens (24, 25), it should be noted that P69, *per se*, as many other vacuolar components, is also deleterious to the plant cell itself. The P69 proteinase causes rapid degradation of many cellular proteins, of which RuBPCase degradation is the most conspicuous (24). Thus, it seems logical that confinement of P69 within a separate cellular compartment (the vacuole) controls its hydrolytic function. Its action would be effective after vacuoles display autophagic activity by engulfing cytoplasm. This concept agrees with the proposals of Matile and Winkenbach (11) and Wittenbach *et al.* (29) and also with the idea proposed by Van der Wilden *et al.* (20), which consider protein bodies as autophagic vacuoles.

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Decompartmentalization of plant cells has been argued to be a response to pathogen attack (2, 22, 28). Accordingly, we envisage that the last steps in viroid pathogenesis would be the disruption of the vacuole with liberation of its hydrolytic constituents, including P69 proteinase, to the intercellular spaces. The way in which the collapse of critical cells leads to the appearance of pathological symptoms is a matter of discussion. Clearly, such a complex process must involve the cooperative interaction of multiple factors and, as with other similarly regulated metabolic events, may be initiated by more than one mechanism. Questions regarding further involvement of P69 proteinase and probably other relevant hydrolases in viroid pathogenesis remain to be answered.

Targeting of P69 proteinase to the apoplast could be a direct process rather than a result of vacuolar disruption. The study of this transport, as well as the isolation of vacuoles and characterization of 'inclusion bodies,' may further our understanding of the biological role of PR proteins.

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