Detection of Pathogenesis Related Proteins Associated with Viroid Infection in Citrus

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ABSTRACT. A pathogenesis-related (PR) protein of approximately 14 kDa has been identified in neutral extracts from Etrog citron infected with citrus exocortis viroid (CEVd) and the citrus viroids of the CVd-I group. The protein has been detected in plants infected with different CEVd sources. Plants infected with CVd-Ia showed similar titers of the protein to plants infected with CEVd, whereas plants infected with CVd-Ib usually showed very low amounts. Western-blot analysis using an antiserum obtained against the 14-KDa protein (P14) from CEVd infected tomato extracts indicated no relationship between the proteins identified from citron and tomato.

No PR proteins were detected from Pineapple sweet orange, a symptomless hosts for citrus viroids, extracted under the same conditions. However, high titers of a 23-kDa protein (P23) were consistently found in acidic extracts from plants infected with CEVd, CVd-I and CVd-IV. Western-blot analysis using an antiserum obtained against the P23 from CEVd-infected tomato plants indicated no relationship between the proteins identified from citron and tomato.

Index words: exocortis, cachexia, xyloporosis, stress

Pathogenensis-related (PR) proteins are a family of host-encoded plant proteins induced under stress situations. A number of PR proteins induced after infection with necrotizing and non-necrotizing viruses have also been described (2). The biological role of these proteins is not well understood but some of them have been characterized as having glucanase, peroxidase, protease or chitinase activities (3,10,11).

The synthesis and accumulation of PR proteins has also been described after systemic infection of *Gynura aurantiaca* and tomato with viroids (6,7). Infection of tomato plants with CEVd resulted in the synthesis of a set of at least ten different cationic PR proteins. Further studies established the localization of some of these proteins within the host cell, as well as their associated enzymatic activity and degradation (13,14,16,19,20).

A study was conducted to identify the induction of PR proteins as a result of infection with the complex of citrus viroids. For this study two citrus hosts were chosen, Arizona 861-S1 Etrog citron which replicates and/or accumulates high titers of most citrus viroids expressing specific symptoms, and Pineapple sweet orange, a symptomless hosts for all citrus viroids (8).

MATERIALS AND METHODS

Viroid sources and hosts. Citron plants propagated on rough lemon rootstock and Pineapple sweet orange seedlings were graft-inoculated with the single viroid sources included in the citrus viroid consensus catalogue (16). The inoculated plants were maintained in a temperature-controlled greenhouse at 28-32 C. Three to six months after inoculation, the citron plants showed the symptoms characteristic of each viroid group. Viroid infection was further tested by nucleic acid extraction and sequential polyacrylamide gel electrophoresis (sPAGE) analysis. Viroid infection on inoculated Pineapple sweet orange seedlings was assessed by graft inoculation on citron followed by sPAGE analysis of nucleic acid extracts three months later (9).

Protein extraction and SDS-PAGE analysis. For protein extraction at neutral pH, 0.5 g of lyophilized leaves were homogenized in 10 ml extraction buffer (0.26 M Tris-H_3PO_4, pH 6.9; 1 mM PMSF; 0.3% 2-mercaptoethanol). Homogenates were centrifuged at 18,000 *g* for 20 min at 4 C and filtered through a cheesecloth (12). Fractions of the supernatant (65 µl) were boiled for 5 minutes in 30 µl of a boiling mixture containing 0.26 M Tris-HCl, pH

8.0; 6% SDS, 15% 2-mercaptoethanol and 30% glycerol, and were subjected to PAGE without further purification.

For protein extraction at acidic pH, 0.5 g of lyophilized leaves were homogenized in 10 ml extraction buffer (84 mM citric acid, 32 mM Na_2HPO_4, pH 2.8; 0.3% 2-mercaptoethanol). Homogenates were centrifuged at 15,000 *g*, and the supernatant fraction concentrated by precipitation in 80% $(NH_4)_2SO_4$. After centrifugation at 18,000 *g* for 20 min the precipitate was resuspended in buffer 0.26M Tris-H_3PO_4, pH 6.9, and dialyzed overnight against the same buffer. Fractions (65 µl) of these preparations were boiled as described above and subjected to PAGE (17,18).

Denaturing SDS-PAGE (14% polyacrylamide in the resolving gel) was performed as described by Conejero and Semancik (5). The low molecular weight markers (Bio-Rad Laboratories) used were: rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), hen-egg white lysozyme (14.4 kDa). Electrophoresis was run at a constant 2 mA intensity per sample until the front marker reached the bottom of the gel. Proteins were visualized by staining the gel with 0.05% Coomassie brilliant blue G 250.

Western blot and indirect ELISA analysis. After SDS-PAGE, proteins were electroblotted on polyvinylidene fluoride (PVDF) membranes (Immobilon-P, from Millipore) using a Milliblot semidry SDE apparatus. A constant voltage with an initial intensity of 2.5 mA cm^{-2} of gel area was supplied using the following buffers: 0.3 M Tris, pH 10.4 (anode buffer No.1); 25 mM Tris, pH 10.4 (anode buffer No.2); 25 mM Tris, 40mM amino-n-capronic acid, pH 9.4 (cathode buffer). The membranes were blocked with TS buffer (50 mM Tris-HCl, 0.3 NaCl, pH 7.5) containing 0.5% Tween-20 during 16 hr at 4 C. The antisera used had been previously obtained against p14 and P23 from tomato (19), and were kindly provided by Prof. V. Conejero (Biotechnology Dept., Universidad Politécnica, Valencia). They were used at 1:500 and 1:250 dilutions for p14 and P23 respectively, and incubated with gentle shaking at room temperature for 2 hr. The membranes were washed four times during 5 min each with TS buffer containing 0.05% Tween-20. Goat anti-rabbit antiserum conjugated with alkaline phosphatase (4) was used at 1 µg/ml in TS buffer and incubated at room temperature for 2 hr with gentle shaking. After four 15 min washings in TS, the reaction was developed by the nitrobule tetrazolium method of Blake, *et al.* (1).

RESULTS

Identification of a 14-kDa protein from inoculated citrons. A protein band with an estimated molecular weight of 14 kDa was identified in electrophoresed extracts from citrons infected with CEVd showing the characteristic stunting, epinasty and necrosis associated with CEVd infection. This 14-kDa protein was always detected from CEVd infected citrons, regardless of the CEVd source (four different sources were tested), but it was absent in extracts from the uninoculated controls (Fig. 1).

A 14-kDa protein was never detected in extracts from plants infected with CVd-II, CVd-III and CVd-IV viroids showing the characteristic symptoms of these viroid groups (Fig. 2). None of the plants infected CVd-II (IIa, IIb, IIc), CVd-III (IIIa, IIIb, IIIc, IIId) or CVd-IV viroids showed the 14-kDa protein (data not shown); however, the protein was detected in extracts from plants infected with CVd-I viroids. Very low amounts of the protein were detected in extracts from plants infected with CVd-Ib as compared with plants infected with CV-Ia (Fig. 3).

Since a protein of 14 kDa had already been described and further characterized from *Gynura aurantiaca* and tomato infected with CEVd (16,19), protein preparations from citrons infected with viroids (CEVd,

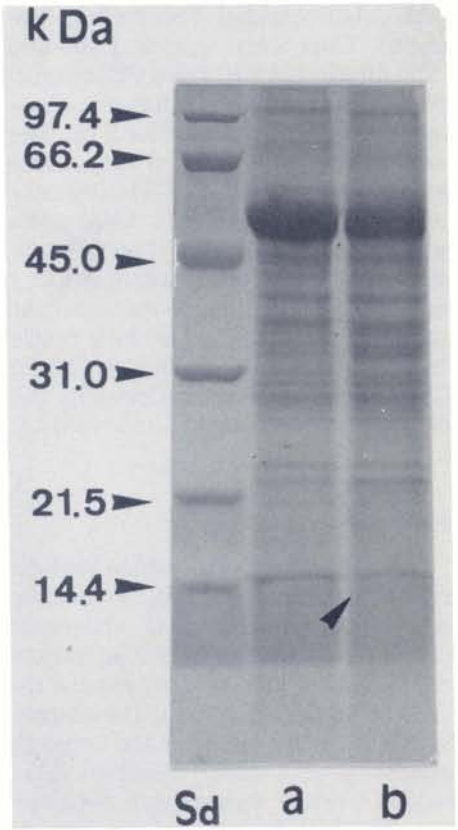

Fig. 1. Fractionation by polyacrylamide gel electrophoresis SDS/PAGE of proteins extracted at pH 6.9 from Etrog citron: a) uninoculated control; b) infected with CEVd. Molecular weight standards (Sd). Arrowheads indicate the 14KDa-protein.

CVd-Ia, CVd-IIa, CVd-IIId, CVd-IV) were separated by SDS/PAGE and subjected to Western blot and indirect ELISA analysis using an antiserum prepared against the protein P1(p14) from tomato. No reaction was observed between the 14-kDa protein induced in citron as a result of infection with CEVd and CVd-Ia viroids and the antiserum against tomato P1 (Fig. 4).

Identification of a 23-kDa protein from inoculated sweet orange. Pineapple sweet orange plants were inoculated with single viroid sources and displayed no symptoms. The inoculated plants tested positive for viroids after bioassay on Etrog citron and nucleic acid extraction and sPAGE. When total proteins were extracted at pH 6.9,

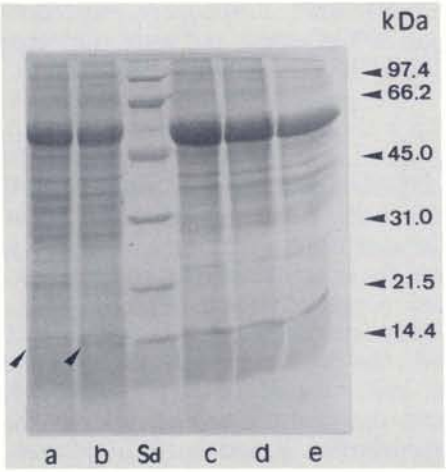

Fig. 2. Fractionation by SDS/PAGE of proteins extracted at pH 6.9 from Etrog citron infected with: CEVd (a); CVd-Ia (b); CVd-IIa (c); CVd-IIId (d); CVd-IV (e). Molecular weight standards (Sd). Arrowheads indicate the 14-dDa protein.

no differences on protein profiles were observed between viroid infected and the uninoculated controls after SDS/PAGE analysis (data not shown). However analysis of proteins extracted at pH 2.8 revealed marked differences between extracts from plants infected with CEVd, CVd-I and CVd-IV vir-

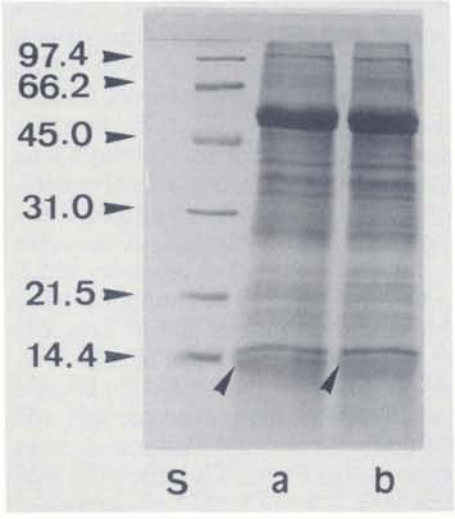

Fig. 3. Fractionation by SDS/PAGE of proteins extracted at pH 6.9 from Etrog citron infected with: a) CVd-Ia; b) CVd-Ib. Molecular weight standards (Sd). Arrowheads indicate the 14KDa-protein.

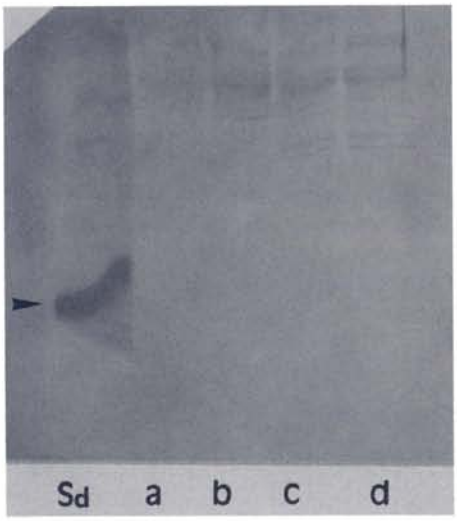

Fig. 4. Western blot and indirect ELISA analysis of proteins extracted at pH 6.9 from citron and fractionated by SDS/PAGE, using a specific antiserum raised against P1(p14) protein from tomato: a) uninoculated control; b) CEVd; c) CVd-Ia; d) CVd-Ib. Preparation of p14 (arrowhead) from tomato was used as an internal control (Sd).

oids as compared with the extracts from uninoculated controls. High titers of a protein of approximately 23 kDa was consistently identified in plants inoculated with CEVd, CVd-I and CVd-IV, as compared with the low titers observed in extracts from plants in-

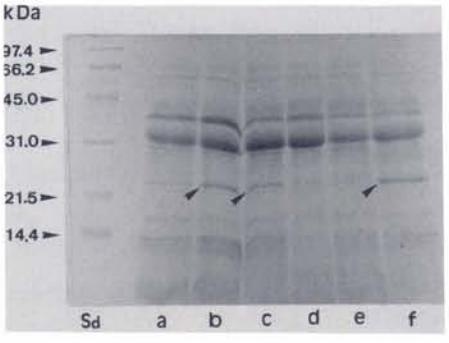

Fig. 5. Fractionation by SDS/PAGE of proteins extracted at pH 2.8 from Pineapple sweet orange infected with CEVd (b); CVd-Ia(c); CVd-IIa(d); CVd-IIId(e); CVd-IV(f), and uninoculated control (a). Molecular weight standards (Sd). Arrowheads indicate the 23-kDa protein.

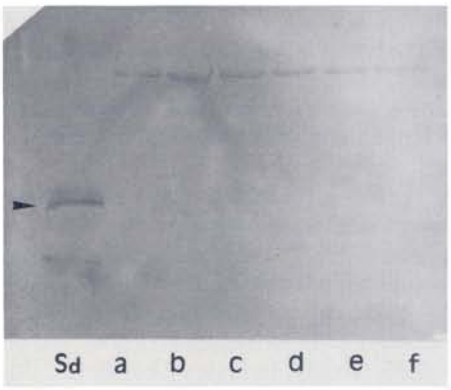

Fig. 6. Western blot and indirect ELISA analysis of proteins extracted at pH 2.8 and fractionated by SDS/PAGE, using a specific antiserum raised against P34 protein from tomato. Protein extracts from Pineapple sweet orange infected with: CEVd (b), CVd-Ia (c), CVd-IIa (d), CVd-IIId (e), CVd-IV (f), and uninoculated control (a). Preparation of P23 (arrowhead) from tomato was used as an internal control (Sd).

fected with CVd-II and CVd-III viroids which were indistinguishable from the uninoculated controls (Fig. 5).

Since a protein of 23 kDa had been previously described from CEVd infected tomatos and further characterized as a thaumatin-like protein associated with osmotic stress (14), the protein preparations from infected sweet orange plants extracted at pH 2.8, were separated by SDS/PAGE and subjected to Western blot analysis using antiserum prepared against the protein P23 from tomato. No reaction was observed between the 23-kDa proteins induced in sweet orange as a result of infection with CEVd, CVd-I and CVd-IV viroids and the antiserum against tomato P23 (Fig. 6).

DISCUSSION

Protein analysis of citrus systemically infected with citrus viroids revealed two PR proteins. A 14-kDa protein has been identified in citron infected with CEVd and CVd-I viroids showing characteristic symptom of these two types of viroids. A-23 kDa protein has been identified in the symptomless host Pineapple sweet orange infected

with CEVd, CVd-I and CVd-IV viroids. No relationship has been found after Western blot analysis between these two proteins and PR proteins P1(p14) and P23 associated with tomato plants infected with CEVd.

Different viroid groups have been associated with specific symptoms in citron (8,9). Therefore the differences observed in terms of induction of pathogenesis-related proteins may reveal differences in pathogenicity among different viroids. In fact, although CEVd is resposible for the most severe symptoms in citron and CVd-I viroids only cause mild leaf epinasty, provided the environmental conditions are adequate both viroids induce curling of the leaf blade as a result of midvein necrosis, symptoms which are not associated with the other viroids grups. The biological significance of these proteins with this kind of symptoms must be further studied.

Although sweet orange is considered a symptomless host for citrus viroids, the identification of high titers of a 23-kDa protein in infected with CEVd, CVd-I and CVd-IV viroids reveals that differences in pathogenicity among different viroids exist even in latent infections. The significance of this protein in pathogenesis and/or defense mechanisms has not been yet determined.

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