Citrus Exocortis Viroid Infection Alters the in Vitro Pattern of Protein Phosphorylation of Tomato Leaf Proteins

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The involvement of protein phosphorylation in the pathogenesis of citrus exocortis viroid (CEV) in tomato (Lycopersicon esculentum) plants was investigated by determining the pattern of protein phosphorylation after labeling with \( \gamma ^{32} \text{P} \)ATP. \( ^{32} \text{P} \) incorporation into proteins was determined by autoradiography after fractionation of proteins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. CEV infection markedly and consistently enhanced the phosphorylation of several proteins with molecular masses of 68, 40, 30, and 27, and a triplet of 22–24 kDa. Also, a reduction in the phosphorylation of other proteins, normally phosphorylated in mock-inoculated tomato plants, was found. The expression of this differential phosphorylation of proteins was Mn\(^{2+}\)-dependent. These viroid-induced effects on protein phosphorylation could only be observed after disease symptoms had emerged in the plant.

Additional keyword: protein kinase.

A distorted pattern of development is a rather common characteristic of most plant diseases irrespective of the nature of the pathogen (Van Loon 1982, 1987; Fraser 1987; Zaitlin and Hull 1987; Goodman et al. 1986). Viroids are low molecular weight (1.1–1.3 \( \times 10^4 \)) coaxially closed circular RNA molecules (for recent reviews see Diener 1987; Riesner and Gross 1985; Keese and Symons 1987), which represent the minimal entities with the recognized ability to replicate and induce disease and host defense reactions (Diener 1987; Vera and Conejero 1988, 1989). Because viroids do not code for proteins (Davies et al. 1974; Semancik et al. 1977), it is conceivable that developmental alterations and biological processes produced upon infection must have originated as a consequence of abnormal signaling in the plant (Semancik and Conejero 1987). An ethylene-mediated cascade of biochemical events leading to disease is triggered and, at the same time, a physiological stage less susceptible to subsequent infections develops (Conejero and Granell 1986; Vera and Conejero 1989). In this regard, it is pertinent to note that the existence in plant cells of a system to recognize, amplify, and transduce internal and external stimuli is being increasingly accepted (Ralton et al. 1987). The importance of the phosphorylation of proteins as a key element in this regulatory network is also gaining acceptance (Ranjeva and Boudet 1987; Feller 1989).

We now report that citrus exocortis viroid (CEV) infection in tomato (Lycopersicon esculentum Rutgers) dramatically alters the normal pattern of in vitro protein phosphorylation.

While this work was in progress, Hiddinga et al. (1988) and Jessen Crum et al. (1988) reported the effect of potato spindle tuber viroid and tobacco mosaic virus infection on protein phosphorylation in crude homogenates of tomato and tobacco. In both cases, the only effect reported was the increase in phosphorylation of a 68-kDa host protein homologous to human double-stranded (ds) RNA-dependent protein kinase (Hiddinga et al. 1988; Jessen Crum et al. 1988).

MATERIALS AND METHODS

Plant material. Tomato plants (L. esculentum) were grown under greenhouse conditions (25–30° C, 16-hr photoperiod) in 20-cm pots and were fertilized at 3-day intervals. The plants in the two- to three-leaf stage were inoculated with purified CEV as described previously (Granell et al. 1987). Control plants were mock inoculated using the same procedure except that buffer was used in place of the CEV inoculum.

Preparation of tomato leaf tissue homogenates. Four upper leaves from mock-inoculated or CEV-infected plants were removed from the plants and immediately frozen in liquid nitrogen. For each experiment, 15 g of frozen leaf tissue was homogenized in 45 ml of grinding buffer (30 mM Tris-HCl, pH 7.7, 5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 0.4 M sucrose, 15 mM \( \beta \)-mercaptoethanol, containing 1 mM phenylmethylsulfonyl fluoride [PMSF] and 1 mM \( p \)-chloromercuribenzoic acid [pCMB] as proteinase inhibitors) in a Polytron (Kinematica GmbH, Lucerne, Switzerland) for 30 s at high speed. The homogenates were filtered through four layers of cheesecloth and subjected to centrifugation at 2,000 \( \times \) g for 5 min to remove nuclei and cell debris. The supernatants were further centrifuged at 16,000 \( \times \) g for 20 min. The resulting supernatants were then divided into 1-ml aliquots and kept frozen at -70° C. All the operations were performed at 4° C.

Phosphorylation of proteins in crude homogenates. Phosphorylation of soluble proteins (16,000 \( \times \) g supernatant) was performed in the following manner. The reaction mixture (final volume of 100 \( \mu \)l) contained 0.1 mg of protein in buffer A (30 mM Tris-HCl, pH 7.7, 5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM PMSF,
1 mM pCMB, and 1 mM MnCl₂. When indicated, MnCl₂ was omitted from this buffer. The phosphorylation was initiated by adding ATP to a final concentration of 10 μM with 10 μCi (γ-³²P)ATP (370 GBq/mmol) (Amersham, Braunschweig, West Germany). Incubation was conducted for 1 min at 37°C, and the reaction was terminated by adding an equal volume of electrophoresis sample buffer (25 mM Tris-HCl, pH 6.8, 6% [w/v] sodium dodecyl sulfate [SDS], 0.1% [v/v] β-mercaptoethanol, and 0.005% [w/v] bromophenol blue) and heating for 5 min in a boiling water bath.

**PAGE and autoradiography.** Proteins were fractionated by electrophoresis on 14% discontinuous SDS-polyacrylamide slab gels (SDS-PAGE) as described previously (Conejero and Semancik 1977). After protein fixation and staining with Coomassie Brilliant Blue R 250, the gels were dried and then exposed to Kodak X-Omat AR films for 12-24 hr with intensifying screens. Autoradiographs were scanned with an LKB 2202 Ultrascan laser densitometer. The incorporation of ³²P into specific proteins was evaluated by measuring the peak area above the background on the densitometric tracing. All gels contained the following molecular mass markers (Sigma, St. Louis, MO): bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14 kDa).

**Western blotting.** Crude protein extracts were subjected to SDS-PAGE and electrophoresed onto nitrocellulose membranes. Protein transfer was performed in a Trans-Blot system (Bio-Rad, Richmond, CA) in 25 mM Tris, 150 mM glycine, 20% (v/v) methanol at 4°C and at a current of 200 mA for 3.5 hr as described by Towbin et al. (1979) with the modifications described elsewhere (Vera et al. 1988). The nitrocellulose membrane was then incubated with mouse anti-human dsRNA-dependent protein kinase p68 antibody (1:40 dilution) for 2 hr at 30°C. The western blots were developed with the Bio-Rad Immunoblot (goat anti-mouse IgG horseradish peroxidase conjugate) assay kit.

The anti-human dsRNA-dependent protein kinase p68 antibody and sample of interferon-treated Daudi cell extracts (containing the p68 kinase) were obtained from Ara Hovanessian (Institut Pasteur, Paris).

Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

**RESULTS**

CEV infection alters the *in vitro* pattern of protein phosphorylation. We sought to demonstrate an effect of CEV infection on protein phosphorylation in tomato leaf homogenates. After labeling with (γ-³²P)ATP and analysis by SDS-PAGE and autoradiography, several polypeptides (68, 40, 30, 27, a faint triplet of 22-24 kDa, and one of 13 kDa) from leaf homogenates of plants infected with CEV showed a higher degree of phosphorylation when

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**Fig. 1.** Effect of citrus exocortic virus (CEV) infection on the protein phosphorylation pattern in leaf homogenates of tomato plants. Crude leaf homogenates from CEV-infected and mock-inoculated tomato plants (25 days postinoculation) were incubated as indicated in the text in buffer A for 1 min with (γ-³²P)ATP. After boiling in sodium dodecyl sulfate (SDS)-electrophoresis sample buffer, proteins (30 μg) were analyzed by SDS-PAGE and autoradiography. A, Densitometric scanning of the autoradiograph shown in C. Relative absorbance of the phosphorylated proteins is indicated at the ordinate. B, Coomassie blue staining of proteins. C, Autoradiograph of the same gel shown in B. Molecular mass marker proteins (kDa) are indicated. P↑(p14) denotes the position of the 14-kDa host protein induced by CEV infection in tomato. LS and SS indicate the large and small subunits of tomato ribulose 1,5-bisphosphate carboxylase (RubPCase). Lanes I and H, proteins from leaf homogenates of tomato plants infected with CEV and of healthy plants, respectively.

**Fig. 2.** Time course of protein phosphorylation pattern induced by citrus exocortic virus (CEV) infection in tomato plants. Tomato plants were inoculated with CEV (I) or with buffer (H) and grown for various lengths of time (0-30 days) postinoculation (p.i.) under greenhouse conditions. At the indicated times, four upper leaves from three different plants were collected, and homogenates were assayed with (γ-³²P)ATP as indicated in Figure 1. Equal amounts of proteins (30 μg) were then analyzed by sodium dodecyl sulfate-PAGE and autoradiography. Filled arrows indicate the proteins specifically phosphorylated in leaf homogenates from plants infected with CEV (I). Open arrows indicate those proteins whose phosphorylation is reduced in leaf homogenates from plants infected (1) with CEV when compared with leaf homogenates from healthy plants (H).
compared with noninfected controls (Fig. 1). In addition, in homogenates from leaf tissue infected with CEV a decrease, if not a suppression, in the phosphorylation of several polypeptides (46, 37, and 32 kDa) was observed, compared with normally phosphorylated proteins from mock-inoculated plants. Coomassie blue staining revealed minor differences in protein content; of these, the protein band corresponding to the previously reported "pathogenesis-related" (PR) P1 protein (Granell et al. 1987; Vera et al. 1989) induced by CEV infection in tomato plants is the most conspicuous.

**Time course study.** To ascertain whether any relationship exists between disease symptom development and protein phosphorylation, a comparative study (CEV-inoculated versus mock-inoculated) was conducted during a period of time starting with the inoculation of plants and ending when plants fully expressed symptoms (30 days postinoculation). An increase in the phosphorylation of the 68-, 40-, 30-, and 27-kDa polypeptides was clearly detected 15 days after inoculation of plants with CEV, concomitant with the onset of disease symptoms, and was of the same order in samples taken at 20 and 30 days after inoculation (Fig. 2). In homogenates from mock-inoculated plants, we could not observe any variation either in the level or in the pattern of the phosphorylated proteins (Fig. 2).

**Effect of various compounds on protein phosphorylation.** The addition of 1 mM MnCl₂ to the incubation buffer enhanced viroid-induced changes in protein phosphorylation (Fig. 3). In fact, the absence of Mn²⁺ in the incubation media completely abolished the phosphorylation of most proteins either in homogenates of healthy or infected leaf tissue. A higher concentration of Mn²⁺ (10 mM) neither enhanced nor inhibited the phosphorylation of these proteins (not shown).

As shown in Figure 4, the addition of unlabeled ATP (300 µM) to the Mn²⁺-dependent kinase assay of crude leaf homogenates from plants infected with CEV completely abolished the ^32P incorporation into proteins. The addition of GTP caused a 10–20% inhibition. CTP and TTP had no effect on the phosphorylation of these proteins. This indicates that the main substrate for the kinase(s) activity is ATP.

To search for any dsRNA-dependent protein kinase possibly present in the extracts being studied (Hiddinga et al. 1988), different synthetic RNA molecules were added to the incubation media. We also included dsDNA molecules as a control for specificity. No enhancement of the phosphorylation of any protein could be found at concentrations of dsRNA molecules of 10 µg/ml, which have been shown to produce activation of dsRNA-dependent kinases (Hiddinga et al. 1988; Galabru and Hovanessian 1987; Galabru et al. 1989) (Fig. 5), or at higher concentrations (10–50 µg/ml; data not shown). In addition, we could not detect any cross-reactivity when crude homogenates from tomato plants were assayed with anti-human dsRNA-dependent protein kinase p68 antibody by immunoblot (Fig. 6). Under the same conditions, on immunoblots of interferon-treated Daudi cell homogenates (containing the dsRNA-dependent kinase p68), this protein was recognized by the antibody (Fig. 6, lane 3).

![Fig. 3. Effect of MnCl₂ on protein phosphorylation in crude homogenates of healthy (mock-inoculated) (H) tomato plants and in crude homogenates of plants infected (I) with citrus exocortis viroid. Homogenates were obtained in buffer A (without MnCl₂) as indicated in the text and incubated either in the absence (−) or in the presence (+) of MnCl₂ (1 mM); (γ-32P)ATP was added to each tube and the reaction continued for 1 min. Thirty micrograms of protein was layered on each lane.](image)

![Fig. 4. Effect of different nucleotide triphosphates on the phosphorylation of proteins in homogenates of tomato plants infected with citrus exocortis viroid. Aliquots of crude homogenates, prepared as described in Figure 1, were incubated with unlabeled ATP, GTP, TTP, or CTP (300 µM final concentration). (γ-32P)ATP was added to each tube, and each tube was incubated at 37°C for 1 min. Phosphorylated proteins were analyzed as described in Figure 1.](image)
DISCUSSION

The data presented here show that CEV infection induces in tomato plants the phosphorylation of several polypeptides with $M_r$ ranging from 24,000 to 68,000. In homogenates from healthy (mock-inoculated) plants, the phosphorylation of these proteins is practically undetectable under the same assay conditions. At the same time, the level of phosphorylation of several polypeptides notably phosphorylated in homogenates from healthy plants is either greatly reduced or abolished in those from the infected plants. The effect of CEV infection on protein phosphorylation is first detected 15 days after inoculation, when the initial symptoms of disease ("leaf-blade" malformations) arise in the plant, and is essentially maintained at similar levels at later stages. The difference on protein phosphorylation is more clearly observed when Mn$^{2+}$ ions are included in the kinase assay buffer. This indicates that the effect of viroid infection is mostly displayed through Mn$^{2+}$-dependent protein kinases.

Our results differ from those reported by Hiddinga et al. (1988) for the system potato spindle tuber viroid-tomato plant. In that work, the only viroid-induced effect reported was the increase in phosphorylation of a 68-kDa protein that was considered homologous to a dsRNA-dependent protein kinase from animals. According to our results, the phosphorylation of the 68-kDa protein was not enhanced by exogenously added dsRNA molecules, and this protein did not cross-react with antibodies against human dsRNA-dependent protein kinase p68. This result differs from the cross-reactivity reported by these authors for a similar 68-kDa phosphorylated protein.

The functionality of the induction of animal dsRNA protein kinase is to shut off host cell protein translation upon virus infection through phosphorylation of eIF-2 (Sickierka et al. 1985; Reichel et al. 1985; Berry et al. 1985; Schneider and Shenk 1987). The arrest of protein synthesis is not observed in viroid-infected tissue (Zaitlin and Harahurampanian 1972). Instead, the infected tissue synthesizes an array of new proteins, called PR proteins (Granell et al. 1987; Vera and Conejero 1988; Camacho-Henriquez and Sanger 1982). Thus, the existence of a dsRNA protein kinase with a reported function such as this does not seem to apply in viroid infection. The apparent discrepancy of our results with those reported by Hiddinga et al. (1988) merits further investigation.

The observed differences in protein phosphorylation when infected with CEV probably reflect changes in the susceptibility of specific proteins to be phosphorylated. This effect could be helped by the presence of activators, so far unknown, of the protein kinase(s) responsible for the observed phosphorylation, as is the case in other biological systems (Leader and Katan 1988).

Currently, little is known about the primary biological events controlling the pathological response of the plants against viroid infection. The existence of an initial cellular stage in the viroid-incited response of the plant, independent of the intercellular organization, has been documented (Bellés et al., in press). Also, the need for intercellular signaling for full expression of the disease has been claimed (Semeneick and Conejero 1987). Protein phosphorylation could be operating in these two stages of the host response.

Fig. 5. Effect of synthetic double-stranded RNA and double-stranded DNA molecules on the phosphorylation of proteins. Leaf homogenates from healthy tomato plants and plants infected with citrus exocortis viroid were prepared as described in Figure 1 and divided into aliquots (30 $\mu$g protein), then 10 $\mu$g/ml (final concentration) of the indicated nucleic acid molecule was added to each tube. (γ-32P)ATP was added and incubation continued for 1 min at 37 °C. Lane C represents a control experiment in which no double-stranded molecules were added.

Fig. 6. Immunoblotting of crude protein extracts tested with anti-human p68 antibody. Extracts from interferon-treated Daudi cells (40 $\mu$g protein) containing the double-stranded RNA-dependent protein kinase p68 (lane 3) and extracts from citrus exocortis viroid-infected and mock-inoculated tomato plants (lanes 1 and 2, respectively) (60 $\mu$g protein) were subjected to sodium dodecyl sulfate-PAGE and proteins were transferred to nitrocellulose. Their cross-reactivity with anti-human p68 kinase antibody was determined as described in the text.
The identification of which proteins act as the target substrates for the protein kinases described, as well as the determination of the effect of this phosphorylation on its function, awaits further study. Determining whether this phosphorylation is part of a cellular antiviral response or a subversion of cellular metabolism that may help virus infection may aid in the understanding of virus pathogenesis.

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LITERATURE CITED


