A VIRUS-INDUCIBLE CUCUMBER ANIONIC PEROXIDASE HAS A SEROLOGICAL COUNTERPART IN DIFFERENT PLANT SPECIES

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Summary. – A highly specific rabbit antiserum raised against anionic peroxidase (PRX) purified from cucumber (*Cucumis sativus* L., *cv.* Laura) cotyledons infected with tobacco necrosis virus (TNV) was used to further investigate the antigenic relatedness of peroxidases in the selected plant species. Immunoblot analysis using native polyacrylamide electrophoresis (PAGE) revealed that the antiserum recognized only fast-moving anionic PRX isoenzymes and that cross- reacting protein bands were present in five plant species belonging exclusively to the *Solanaceae* family. There was no serological cross-reaction indicated in virus-infected plant species from three other dicotyledonous families (*Amaranthaceae, Chenopodiaceae and Leguminosae*). Marked qualitative, as well as quantitative differences in the degree of serological relatedness were apparent.

Key words: cucumber; immunoblot analysis; peroxidase isoenzymes; tobacco necrosis virus.

Introduction

Virus infection can have a dramatic effect on its plant host and frequently releases a cascade of events resulting in the disease syndrome. Many plants show a hypersensitive response (HR) to virus infection, which often results in necrotic local lesions at the primary infection sites (Ponz and Bruening, 1986). This type of defense response is accompanied by the triggering of a wide range of inducible defense genes coding for a number of defense-related proteins (for review see Bowles, 1990). However, the best-known group of infection-, or a more broadly, stress-induced plant proteins is formed by the diverse classes of pathogenesis-related (PR) proteins (Bol *et al.*, 1987; Bowles, 1990). Among them the peroxidases have been studied extensively in higher plants for many years (Gaspar *et al.*, 1982; Greppin *et al.*, 1986; Penel *et al.*, 1992; Wellinder *et al.*, 1993).

An acidic PRX is induced and rapidly accumulated in the cotyledon tissue of the cucumber (*Cucumis sativus* L., *cv.* Laura), reacting hypersensitively to infection with TNV; it is absent from the comparable healthy cotyledons (Repka *et al.*, 1991). This PRX consists of at least three serologically related molecular forms of slightly different molecular mass (Repka and Slováková, 1994). In previous observations (Repka and Vanek, 1993), it was indicated that beside the virus infection this PRX was induced by various biotic and abiotic stimuli. Subsequently, an organ-specific pattern of expression was demonstrated using a highly specific anti-PRX serum (Repka and Jung, 1995), prepared against the purified PRX proteins (Repka and Slováková, 1994).

To obtain a more exact idea about the potential role(s) of these PRXs in diseased plant, we attempted to ascertain whether proteins related to cucumber PRX are induced in divergent plant species upon TNV inoculation. Moreover, we describe the use of immunoblot analysis in the investi-

Abbreviations: HR = hypersensitive response; ICF = intracellular fluid; PAGE = polyacrylamide gel electrophoresis; PR = pathogenesis-related; PRX = peroxidase; SDS = sodium dodecyl sulphate; TNV = tobacco necrosis virus

gation of the extent of homology of these PRXs by determining their antigenic relatedness to the cucumber PRX.

Materials and Methods

Plant material. Cucumber plants (*Cucumis sativus* L., *cv.* Laura) were raised in 10 cm pots containing a sterilized standard potting compost in a greenhouse at temperature maintained between 20 – 30°C. Other plant species used (for a complete list see Table 1) were grown in a perlite-peat compost in a greenhouse at the same temperature regime as described above for 2 to 3 months after sowing. All plants were watered daily and fertilized weekly with Substral (Henkel, Bratislava, Slovakia).

Inoculation of plants. Cotyledons of about 7-day-old cucumber plants were abrased using carborundum and then infected with partially purified suspension of TNV as described previously (Repka and Slováková, 1994). *Solanaceous* plants were inoculated with TNV when 8 to 12 weeks old. Seven-week-old and 8-week-old *Chenopodium quinoa* and *Amaranthus cruentus*, respectively, were similarly inoculated with TNV. *Vigna unguiculata* plants were inoculated at the eight-leaf stage with TNV and the resulting necrotic areas were removed for extraction after 7 days. Leaves of control plants were inoculated similarly with virus isolation buffer.

Protein extraction. Water-soluble extracellular proteins were extracted from cotyledons and leaves by the vacuum infiltration procedure as described in detail elsewhere (Repka *et al.*, 1993). The intercellular fluid (ICF) thus obtained was centrifugally concentrated using a Microcon-3 microconcentrator (Amicon, Witten, FRG) following the protocol recommended by the supplier. The samples were used immediately or freeze-dried and stored at -20°C.

Discontinuous PAGE (10% gel) of acidic proteins was performed at 4°C under native conditions according to Laemmli (1970), with the exception that sodium dodecyl sulphate (SDS) was omitted from all buffers and the sample buffer did not contain ß-mercaptoethanol. Each lane was loaded with equal amount of proteins and Bromphenol Blue as a marker. PRX isoenzymes in gels were visualized by activity staining using 0.03% hydrogen peroxide and 0.03% 3,3-diaminobenzidine as the hydrogen donor, at pH 7.0 (Repka and Slováková, 1994).

Protein concentration in samples was estimated with the Bradford (1976) reagent using bovine serum albumin as the standard.

Immunoblot analysis. Proteins separated on native polyacrylamide gels were electroblotted at 4°C for 20 hrs at 50 mA to a nitrocellulose membrane (Protran BA-85, 0.45 µm, Schleicher and Schuell, Dassell, FRG) with 0.7% acetic acid as blotting buffer. Following the transfer, the membrane was baked at 80°C for at least 8 hrs to inactivate endogenous PRX activity. The membrane was blocked in Blotto solution containing 5% non-fat dry milk and further processed basically as described previously (Repka and Slováková, 1994). For obtaining a complex picture about the presence of individual PRX isoenzymes, a mixture of cucumber anti-PRX 1, 2 and 3 antibodies was employed.

Densitometry. Protein bands on the dried gels stained for PRX and on the immunoprocessed blots were estimated using a computerized densitometer GDS 5000 (UVP Products, Cambridge, UK).

Results

Comparison of PRX zymograms of selected plant species

To separate and assay the anionic PRX isoenzymes, total water-soluble proteins extracted from TNV-infected leaves of the selected plant species were subjected to high-resolution native PAGE. The activity staining (Fig. 1A), as well as the computer densitometry analysis (Table 1) clearly demonstrated that there was none or a very little anodic PRX activity present in the extracellular spaces of *A. cruentus* or *C. quinoa* and cowpea leaves, respectively. In other plant species tested, the similarity of the PRX zymograms was obvious, particularly with respect to the fast-moving anionic group of PRX bands. Notably, except for the presence of the fast-moving anionic PRX bands in the ICF extracts,

Table 1. Total anionic PRX activities in leaves from selected TNV-infected plant species expressed as a number of isoenzymes detected by computer-assisted densitometry

Plant species	Code	Total number of detected	
		anionic PRX	fast-moving PRX
Family *Solanaceae*			
Datura stramonium (thorn-apple)	DST	9	6
Nicotiana tabacum-xanthi	NTX	5	5
Nicotiana clevelandii	NCL	6	6
Petunia hybrida	PHY	8	4
Lycopersicon esculentum (tomato)	LES	9	6
Family *Chenopodiaceae*			
Chenopodium quinoa	CQA	1	0
Family *Amaranthaceae*			
Amaranthus cruentus	ACR	0	0
Family *Leguminosae*			
Vigna unguiculata	VUN	2	2
Family *Cucurbitaceae*			
Cucumis sativus	CUC	9	6

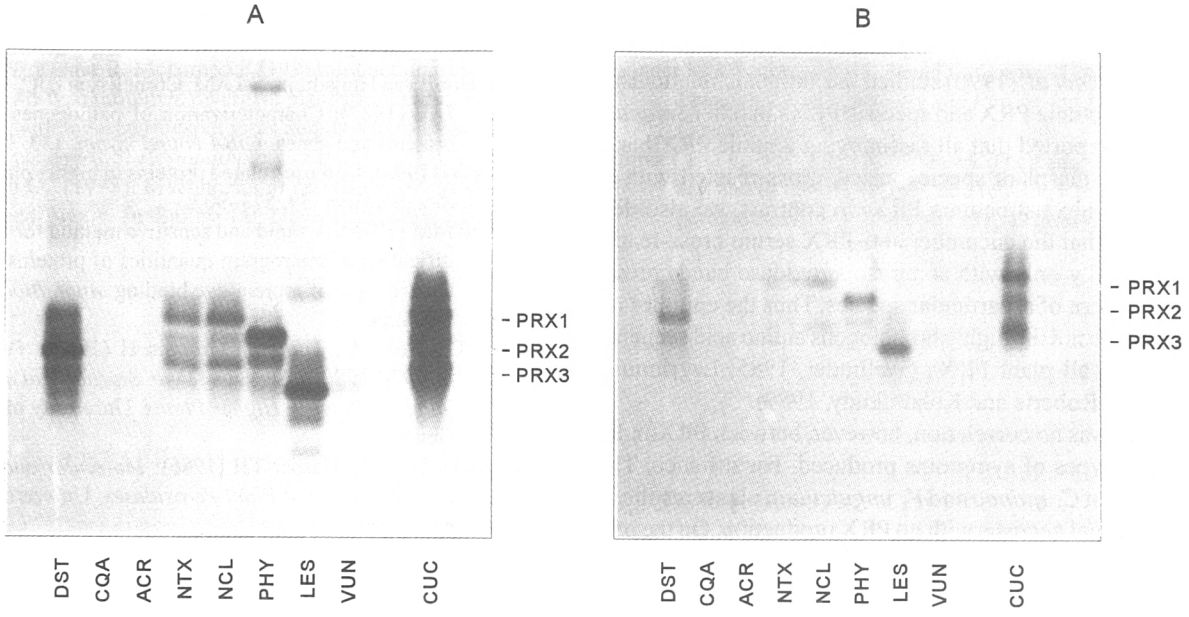

Fig. 1

PAGE patterns of anionic PRXs in extracellular extracts from selected TNV-infected plant species

Polyacrylamide gels ran under native conditions at 4°C were stained for PRX activity with 3,3-diaminobenzidine (A) or subjected to immunoblot analysis using the antiserum against the cucumber PRX (B). Each lane was loaded with 50 µg (A) or 100 µg (B) of proteins. For the codes of the species tested see Table 1. PRX1-3 refer to the three stress-related anionic peroxidase isoenzymes accumulated in TNV-infected cucumber cotyledons.

a marked variability in the number and intensity of these bands was also observed (Fig. 1A, Table 1).

Immunological relationships

The immunoblot analysis using the antiserum against cucumber PRX revealed that the antiserum did not cross-react with the slowly-moving PRXs separated in the upper part of the gel (Fig. 1B). On the other hand, immunoreactive signals only of the fast-moving anionic PRX were apparent.

Using the immunoblot procedure, extracts from 9 plant species representing 5 families were tested for a possible serological cross-reactivity. Surprisingly, beside the extract from TNV-infected cucumber cotyledons, the antiserum gave a positive signal exclusively with the *Solanaceous* species. No specific reaction occured with extracts from the three non-*Solanaceae* species, although they reacted hypersensitively to TNV-infection (Table 2).

In all five *Solanaceous* species the antiserum recognized a set of extracellular PRXs with differential degree of specificity. The most intense signals occured in thorn-apple, petunia and tomato, and to a lesser extent in both tobaccos. Furthermore, it was interesting to see that the bands of the same intensity after the activity staining gave a very different patterns of cross-reactivity with the antiserum against TNV-induced cucumber PRX.

Table 2. Symptoms produced in plants infected with TNV and serological cross-reaction of proteins extracted from these plants with the antiserum against cucumber PRX as determined by immunoblot analysis

Species code	Symptoms	Cross-reaction [a]	No. of reacting isoenzymes[b]
DST	necrotic local lesions	+	4
NTX	brown necrotic lesions	+	3
NCL	necrotic lesions and systemic mosaic	+	3
PHY	small necrotic lesions	+	2
LES	yellow ring spots	+	1
CQA	brown necrotic local lesions	–	–
ACR	leaf curling	–	–
VUN	dark brown local lesions	–	–
CUC	redish-brown necrotic lesions	+	3

[a] Ascertained by immunoblot analysis.
[b] Evaluated by computer-assisted densitometry.

Discussion

Fast-moving anionic PRX isoenzymes serologically homologous to cucumber virus-inducible PRX were induced in most plant species that developed necrosis following TNV infection. Our results demonstrate that the antiserum proved to be specific for cucumber PRX and reacted only with ho-

mologous PRX isoenzymes in the *Solanaceous* plant species.

Hendriks *et al.* (1990) studied the antigenic relationships between petunia PRX and specific PRXs in other *Solanaceae*. They reported that all fast-moving anionic PRX bands, present in the plant species tested, cross-reacted with the antiserum against petunia PRX. In contrast, we also demonstrated that the cucumber anti-PRX serum cross-reacted differentially only with some isoperoxidase bands present in the pattern of a particular species. Thus the epitopes recognized are not the highly homologous amino acid sequences present in all plant PRXs (Wellinder, 1985; Lagrimini *et al.*, 1987; Roberts and Kolattukudy, 1989).

There was no correlation, however, between PRX induction and types of symptoms produced. For instance, TNV infection of *C. quinoa* and *V. unguiculata* plants resulted in a rapid lethal necrosis with no PRX production. On the other hand, the plant species reacted to TNV inoculation only with the production of mosaic or chlorosis (generally non-necrotizing), *e.g.* tomato, produced the immunoreactive PRX. It is very interesting that similar type of response was typical for other PR-proteins in bean plants infected with tobacco ringspot virus (Sehgal *et al.*, 1991).

In this context, there are several acceptable explanations, none of which being mutually exclusive. (1) The absence of virus inducible anionic PRX in necrotizing plant species could be explained as a consequence of rapid collapse and cell death. The PRX fails to accumulate in these species to a detectable level or is not synthesized at all. (2) In these species, some of the basic PRX isoenzymes may be related to this process.

Inversely, the presence of the virus-inducible anionic PRX in non-necrotizing plant species corroborate the generally accepted theory that PRX accumulation represents only a part of complex biochemical machinery associated with the localization of a virus (Van Loon, 1983). It is important to note, however, that the necrosis is not directly responsible for virus localization. Taken together, from these results it is apparent that PRX production and tissue necrosis are independent events.

Induction of proteins related to virus-inducible cucumber anionic PRX in diverse plant species underlines similar homeostatic adjustments in response to different environmental stimuli. Our results presented in this work prove that proteins analogous to the cucumber PRX are synthesized in unrelated species upon viral infection.

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