IMMUNOLOGICAL IDENTIFICATION OF A BASIC HOMOLOGUE TO ACIDIC VIRUS-INDUCIBLE CUCUMBER PEROXIDASE

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Summary. – Eight basic, fast-moving pathogenesis-related peroxidase PR-PRX isoenzymes were found to accumulate in cotyledons of cucumber (*Cucumis sativus* L., *cv.*Laura) reacting hypersensitively to tobacco necrosis virus (TNV). These isozymes were provisionally designated a, b, c, d, d_1, e, f, and g in the order of decreasing electrophoretic mobility in native cathodic polyacrylamide gels. Besides the differential mode of compartmentalization, some of these isoenzymes serologically cross-reacted with the antiserum prepared against the most prominent and best characterized cucumber PR-protein, an anionic PR-PRX. The immunoblot analysis confirmed that, by analogy with the tobacco PR-proteins 1, acidic and basic PR-PRXs also belong to a family of differently charged isomers.

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

Introduction

A disease resistance involves not only a static protection, but also inducible defense gene systems. Although the genetics, cytology and physiology of these plant – pathogen interactions have been extensively studied, until recently relatively little was known at the biochemical level about how plants respond to infection to activate these defense mechanisms. Recent studies have shown that a pathogen attack and/or a fungal elicitor causes massive changes in the pattern of host RNA and protein synthesis, including an accumulation of host-synthesized phytoalexins, cell-wall hydroxyproline-rich glycoproteins (extensins), and hydrolytic enzymes such as chitinases and β-1,3-glucanases (Lawton and Lamb 1987; Benhamou *et al.*, 1991; Niebel *et al.*, 1993; Rasmussen *et al.*, 1992; Wyatt *et al.*, 1991; Repka *et al.*, 1997a). Beside these defense-related products PRXs are generally believed to be important in plant resistance mechanisms by playing an integral role in polysaccharide cross-linkages with extensin monomers (Lamb *et al.*, 1986; Showalter *et al.*, 1986), as well as in the biosynthesis of plant cell wall components including lignin and suberin (Graham and Graham 1991; Roberts *et al.*, 1988).

Likewise for other PR-proteins, namely for hydrolytic enzymes (chitinases and glucanases), there is a high isoenzyme polymorphism in virus-inducible anionic PRX (Repka and Slováková, 1994). This anionic PRX seems to be a representative of a group of fast-moving anionic PRXs present either in *Solanaceae* (Repka, 1996) or *Cucurbitaceae* (Repka, 1997) family. Initially it was thought that all PR-proteins were acidic proteins with low isoelectric points. This was the main reason why the ethylene-inducible, basic bean chitinase was thought not to be a PR-protein (Boller *et al.*, 1983). However, more recently, a plethora of basic PR-proteins have also been described in tobacco and other plants using two-dimensional polyacrylamide gel electro-

Abbreviations: HR = hypersensitive response; EDTA = ethylenediamine tetraacetic acid; ICF = intercellular fluid; PAGE = polyacrylamide gel electrophoresis; PR = pathogenesis-related; PRX = peroxidase; PR-PRX = pathogenesis-related PRX; PVP = polyvinylpyrrolidone; TNV = tobacco necrosis virus

phoresis (PAGE) (Hogue and Asselin, 1987; Joosten *et al.*, 1990; Repka *et al.*, 1993). As these basic PR-proteins seem to be related serologically and/or by sequence homology to their acidic counterparts they may be classified in the same groups. Therefore, to investigate whether, by analogy with the tobacco PR-proteins 1, PR-PRX consists of a family of differently charged isomers with similar molecular characteristics, the antibodies raised against the purified predominant anionic PR-PRX were employed to study basic PR-PRX isoenzymes in more detail.

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 maintained at 20 – 30°C. All plants were watered daily and fertilized weekly with Substral (Henkel, Bratislava, Slovakia).

Inoculation of plants. Cotyledons of cca 7-day-old cucumber plants were abrased using carborundum and then infected with partially purified suspension of TNV (Repka and Slováková, 1994). Leaves of control plants were inoculated similarly with virus isolation buffer. Leaf samples were harvested at daily intervals during seven consecutive days and frozen immediately at -20°C

Protein extraction. Water-soluble extracellular proteins were extracted from cotyledons and leaves by the vacuum infiltration procedure (Repka *et al.*, 1993). The intercellular fluid (ICF) thus obtained was centrifugally concentrated using a Microcon-3 microconcentrator (Amicon, Witten, FRG) following the manufacturer's protocol. The leaves from which the ICF was eluted were used to obtain extracts depleted of ICF. The leaves were ground in a prechilled mortar with pestle with about 1 g of quartz sand and an appropriate amount of Trisepac buffer (50 mmol/l Tris.HCl pH 8.0, 500 mmol/l sucrose, 1 mmol/l ethylenediamine tetraacetic acid (EDTA), 0.2% polyvinylpyrrolidone (PVP), 6 mmol/l ascorbic acid and 0.1% cysteine). The extracts were centrifuged at 15,000 x g for 20 mins at 4°C. The same procedure was used for freshly harvested cotyledons in obtaining total plant extracts. The samples were used immediately or freeze-dried and stored at -20°C.

PAGE and stainig of PRXs. Discontinuous PAGE (10%) of basic proteins was performed at 4°C under native conditions following the original protocol of Reisfeld *et al.* (1962). Each lane was loaded with an equal amount of proteins and a small drop (5 µl) of 5% solution of basic fuchsin was used as tracking dye.

PRX isoenzymes on gels were visualized by activity staining using hydrogen peroxide (0.03%) and 3-amino-9-ethylcarbazole (0.03%) as the hydrogen donor at pH 5.2 (Repka and Slováková, 1994).

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

Immunoblot analysis. Proteins separated by PAGE in native conditions were electroblotted at 4°C for 20 hrs at 50 mA onto nitrocellulose membrane (Protran BA-85, 0.45 µm , Schleicher and Schuell, Dassell, FRG) in a buffer containing 0.7% acetic acid. Then the membrane was baked at 80°C for at least 8 hrs to inactivate endogenous PRX activities, and blocked in a solution containing 5% non-fat dry milk (Blotto). The immunoblots were processed basically as described previously (Repka and Slováková, 1994). To obtain 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 activity-stained gels and immunoprocessed membranes were estimated using a computerized densitometer UVP GDS 5000 (UVP Products, Cambridge, UK) equipped with the powerfull protein analysis software.

Results

Identification of basic PR-PRX isoenzymes

To characterize basic PR-PRXs of cucumber in greater detail, total soluble protein preparations from non-infected and TNV-infected cotyledons were analyzed by cathodic PAGE. Fig. 1A shows the activity-stained separation profiles of basic PRX isoenzymes present in the fractionated plant extracts. Substantial differences were apparent between healthy and virus-infected plants. As a consequence of infection, 8 basic bands designated a, b, c, d, d_1, e, f, and g, corresponding to 8 basic PRX isoenzymes, were observed.

The different virus-inducible basic PR-PRXs were further investigated by immunoblot analysis using the antiserum prepared against the predominant anionic PR-PRX of cucumber. The immunoblots from leaf homogenates showed strongly positive signals in 4 bands only (Fig. 1B, lanes C,D,E). It was also evident that the isoenzyme d_1, detected as a very faint band on the activity stained gel gave very strong signal on the immunoblot (Fig. 1A, lane C and Fig. 1B ,lane C).

Localization of basic PR-PRX isoenzymes

The localization of the different basic PR-PRX isoenzymes was studied in more detail by comparing the pat-

Table 1. A catalogue of total basic PR-PRX isoenzymes in cotyledons of TNV-infected cucumber, type of compartmentalization of individual isoenzymes and cross-reactivity with anti-PR-PRX serum expressed as a number of isoenzymes detected by computer-assisted densitometry

Isoenzyme	Compartmentalization	Cross-reaction
a	ICF	+
b	TE	–
c	ICF	–
d	TE	+
d_1	TE	+
e	ICF	+
f	ICF	–
g	ICF	–

ICF = intercellular fluid (apoplast); TE = total extract (intracellular).

terns of distribution of individual isoenzymes present in fractionated plant extracts 7 days after infection (Fig. 1A, lanes C,D,E). Isoenzymes b,d, and d_1 were primarily localized intracellularly since specific staining of these bands was observed either in the total plant extract or in the ICF-depleted one. On the other hand, isoenzymes a, c, e, f, and g were present strictly in the ICF-fraction, confirming their extracellular compartmentalization (Table 1).

Antibodies raised against the anionic PR-PRX confirmed the compartmentalization of at least four basic isoenzymes (a, d, d_1, and e), while no cross-reactions with other isoenzymes occured (Table 1).

Time-course of the accumulation of different basic PRX isoenzymes in the apoplastic space

The amounts of the six apoplastically localized basic isoenzymes were related directly to the lesion density on the cotyledons and increased with the duration of infection (Fig. 1C). The immunoblot analysis of the cathodically separated ICF-extracts in PAGE under native conditions confirmed the identity of the four PRX bands primarily destinated to this compartment (Fig. 1D). In contrast, the isoenzymes f and g were also acumulated in a time-dependent manner, and, unfortunately, no positive cross-reactions with the anti-anionic PR-PRX serum occurred (Fig. 1D).

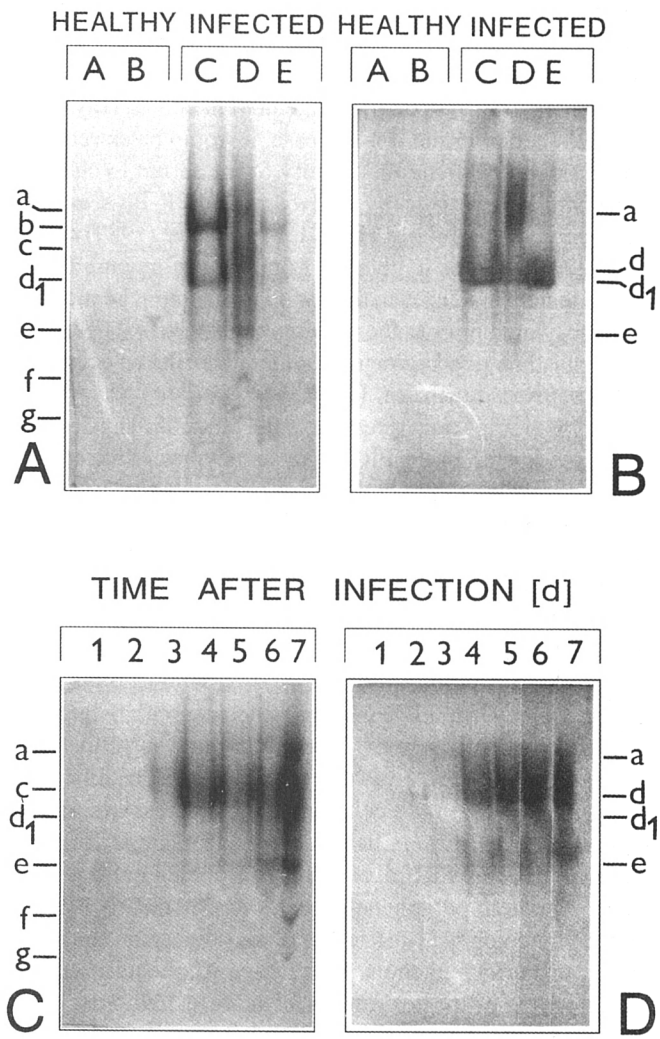

Fig. 1.
PRX activity (parts A,C) and immunoreactivity (parts B,D) of materials separated by cathodic PAGE
Parts A and B: Homogenates of healthy (lanes A and B) and virus infected (lanes C,D and E) cucumber cotyledon tissues. Samples (15 µg protein/lane) of total plant extract (lanes A and C), ICF fraction (lanes B and D) and ICF-depleted homogenate (lane E) were electrophoresed. Immunoblots (parts B and D) were processed with the antiserum against the predominant anionic PR-PRX. Parts C and D: Time course (1-7 days after infection) of the accumulation of apoplastically localized basic PR-PRX isoenzymes.

Discussion

Eight major basic PR-PRX isoenzymes in cotyledons of TNV-infected cucumber were identified. These proteins were not present in healthy plants and, like the previously characterized anionic PR-PRXs (Repka *et al.*, 1993; Repka and Slováková, 1994), accumulated in large amounts after infection.

PAGE and the activity staining of proteins from fractionated plant extracts revealed the presence of at least two main groups of basic PR-PRX isoenzymes, those localized intracellularly and apoplastically (Table 1). To confirm the final destination of these isoenzymes, we used the highly specific antiserum raised against the most prominent anionic PR-PRX. In this context, two kinds of results were obtained from the immunoblot analysis with this serum and fractionated plant extracts from virus-infected cucumber plants. First, the antiserum used recognized specifically an epitope (s) presented by serologically-related counterparts among the acidic and basic PR-PRX isoenzymes. Second, basic isoenzymes localized both intracellularly as well as extracellularly cross-reacted with the respective antiserum. This type of response was quite surprising since the antiserum, originally prepared against the extracellular type of PR-PRX, cross-reacted specifically just with the corresponding antigen, while no reactivity was observed with some of intracellularly localized anionic PRX isoenzymes (Repka, unpublished results).

To date nothing is known about the principle of the cross-reactivity of the anti-anionic PR-PRX serum with its serologically-related basic counterparts. Since the inhibition of the active site is even more isoenzyme-specific, the cross-reactivity may be probably due not so much to the similari-

ties in the primary structure (Stephan and Van Huystee, 1981) as to the similarities in the glycosidic side chains which are, as in other plant glycoproteins (Evans *et al.* 1998; Key and Weiler, 1988), strongly antigenic (Hu and an Huystee, 1988). Whether or not it is the case, it would be extremely interesting to examine the affinity of the serum to chemically and/or enzymatically deglycosylated PR-PRX isoenzyme proteins. Similarly, Hu and Van Huystee (1989) demonstrated that some antibodies from the anti-anionic PRX serum lost their affinity to the basic isoenzymes chemically deglycosylated prior to the immunoblot analysis. The same concept is in a good agreement with the results on extensin (Stafstrom and Staehelin, 1988).

The next important question is the potential physiological function of basic PR-PRX isoenzymes in diseased plants, as well as the significance of serological relatedness between acidic and basic isoforms of prPRX. So far little information about the physiological function(s) of basic PRX is known. Zheng and Van Huystee (1991) demonstrated that the cationic PRX which was highly enriched in the spent medium from peanut cell culture effectively oxidized tyrosine to dityrosine, isodityrosine and polytyrosine. Since basic PRX was found to accumulate largely in the extracellular space (Van den Berg and Van Huystee, 1984), it is suspected to have a close relationship with events occurring in the cell wall, such as crosslinking of extensin. Furthermore, Chabanet *et al.* (1993) reported that cell wall cationic PRX also displays a phenol oxidase activity. Although polyphenol oxidase has no clearly established physiological function(s), it participates in the oxidation of various phenols, especially o-diphenols. Reaction products of the enzyme could serve as hydrogen carriers in the oxidation of various substrates in biological systems (Sherman *et al.*, 1991). The phenomena discussed above also pose question of potential phenol oxidase activity at least of some of basic PR-PRX isoenzymes identified in our work.

A further point of interest concerns the existence of serological relatedness between acidic and basic PR-PRX isoenzymes of cucumber. From functional point of view, it was interesting to see that the expression of both types of antigens (acidic and basic PR-PRXs) was co-localized on tissue print immunoblots (Repka *et al.*, 1997b). It suggests that the both types of PR-PRX isoenzymes could act and/or participate in the same physiological process in diseased plants. Because there is a plethora of examples that PRX is generally important in plant resistance mechanisms, the above mentioned suggests the possible involvement of the studied PR-PRX isoenzymes in imparting the resistance.

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