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BRIEF COMMUNICATION

Immunohistochemical localization of the stress-related anionic peroxidase in germinating cucumber seeds

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Abstract

The distribution of the stress-related anionic peroxidase in the course of cucumber (*Cucumis sativus* L.) seed germination was determined by tissue printing and immunoblotting. Of the three molecular forms of cucumber stress-related anionic peroxidase, the form PRX 1 was temporally accumulated in developing seedlings. Up to 6 d of germination PRX 1 was localized mainly in roots. As germination progressed, the immunoreactive PRX 1 signal was found in the transition zone between roots and stem, as well as in the lower epidermis of expanding cotyledons at the midrib.

Additional key words: *Cucumis sativus*, immunoblotting, tissue printing.

A number of functions for peroxidase and specific isoforms of the enzyme during development, growth and defense have been suggested (Van Huystee 1987, Bowles 1990). To date, however, most of the information available on changes in peroxidase gene expression is indirect and predominantly involves changes in isoenzyme patterns (Lagrimini and Rothstein 1987, Hendriks and Van Loon 1990, Zheng and Van Huystee 1992, Mathur and Vyas 1995).

Leaving aside the biosynthetic and degradative functions carried out by these isoenzymes, there is little information available concerning their precise localization on the organ, tissue or cellular level (Griffing and Fowke 1985, Schloss *et al.* 1987, Ros Barcelo *et al.* 1991). Since plant peroxidases are highly polymorphic and,

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Abbreviations: DAB - 3,3-diaminobenzidine; 1/2 MS - half strength Murashige and Skoog medium; HRP - horseradish peroxidase, PRX - peroxidase, PVP - polyvinylpyrrolidone; TNV - tobacco necrosis virus.
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apparently each isoenzyme has a distinct subcellular localization, enzyme cytochemical probes are insufficient in most cases to establish the exact subcellular localization of the particular isoenzyme. Therefore, the most appealing approach for such study is to employ immunological assays.

Cucumber stress-related anionic peroxidase consists of three molecular forms (termed PRX 1, 2 and 3) of slightly different molecular mass (Repka and Slováková 1994) It was previously demonstrated that the peroxidase is strongly induced and rapidly accumulated in cucumber cotyledons reacting hypersensitively to infection with tobacco necrosis virus (Repka *et al.* 1993). Moreover, we have also shown that beside virus infection the same set of peroxidase isoenzymes was induced by various environmental stimuli (Repka and Vanek 1993). Subsequently, using a highly specific rabbit antiserum raised against this peroxidase from cucumber, it was demonstrated that the peroxidase is differentially expressed also in normal plant development by an organ-specific manner (Repka and Jung 1995).

Here we survey the localization of the PRX 1 isoenzyme in the course of germination of the plant by immunoblotting and tissue printing.

Seeds of *Cucumis sativus* L. cv. Laura were surface sterilized in *Domestos* (*Henkel*, Bratislava, 5 % NaOCl) for 25 min, and soaked for 2 h in sterile distilled water. To germinate, seeds were transferred on to 1/2 Murashige and Skoog (MS) medium saturated *Whatman No. 1* filter paper in Petri dishes and then placed in a humid chamber. Seedlings were grown under 12-h photoperiod, irradiance of 130 W m^{-2} and day/night temperature of 27 - 22 °C and relative air humidity 60 %.

Freshly harvested material was ground rapidly in a prechilled mortar at 4 °C in appropriate volumes of TRISEPAC buffer (50 mM Tris-HCl, pH 8.0, 500 mM saccharose, 1 mM EDTA, 0.2 % insoluble PVP, 6 mM ascorbic acid and 0.1 % cysteine) according to Repka and Slováková (1994). Extract from TNV-inoculated cucumber cotyledons was prepared and concentrated as mentioned elsewhere (Repka and Fischerová 1996)

Protein concentrations were determined by the method of Bradford (1976), and 25 μg of total protein per lane was loaded onto each of a pair of replicate native polyacrylamide gels (4 % stacking, 10 % resolving).

After electrophoretic separation under native conditions the proteins in one gel were electroblotted onto 0.45 μm nitrocellulose (*PROTRAN BA-85, Schleicher and Schuell GmbH*, Dassel, FRG). The other gel was activity stained for anodic peroxidase using 3-amino-9-ethyl carbazole (*Amresco Inc.*, Solon, USA). As negative and positive controls, each blot included a lane containing 25 or 15 μg of total proteins extracted from dry seeds or TNV-inoculated cotyledons, respectively.

Following the transfer, the membrane was baked at 80 °C for at least 8 h to inactivate endogenous peroxidase. Blots were blocked 1 h in 1 × TEN buffer (50 mM TRIS-HCl, pH 7.4, 5 mM EDTA and 150 mM NaCl) containing 5 % dry milk (*Blotto*); and then incubated 1 h with the primary antibody PRX (Repka and Slováková 1994) diluted 1:150 in the blocking buffer. Blots were washed 3 × 10 min in 1 × TEN, 1% Tween 20, incubated 1 h with 1:1000 diluted swine anti-rabbit IgG HRP-conjugated secondary antibodies (*Sevac*, Prague, Czech Republic), and washed

again for 4 × 10 min with 1 × TEN buffer plus 1 % Tween 20. Antibody detection used 0.3 % DAB (*Sigma*, Deisenhofen, Germany) and 0.3 % H_2O_2.

For tissue printing, a modifcation of standard procedures (Cassab and Varner 1987) was used. Fresh hand-sections were pressed for 15 to 20 s onto 0.45 μm *PROTRAN* nitrocellulose membrane where the pretreatment step with 0.2 M $CaCl_2$ was omitted. At least three replicate prints were made with the same-age material. Results were consistent between experiments and among the duplicates in a given experiment. Prints were immunoprocessed as Western blots mentioned above. Controls included no antibody (blocking buffer substituted) or secondary antibody only. One set of tissue prints was stained with Amido black for proteins by incubating for 1 min in 0.1 % solution of the dye.

Immunolocalization in tissue prints was visible after a longer incubation in DAB than was needed for the corresponding Western blots. For a given material, antibody-treated tissue prints were handled identically throughout antibody incubation, HRP development, and photographic processing.

Tissue prints were photographed with *Kodak Gold* 200 ASA film (*Eastman Kodak Co.*, Rochester, USA) using *AO* stereomicroscope *M570* (*American Optical Co.*, Buffalo, USA). Black and white prints were made using *Kodak Multigrade* paper.

The stress-related anionic peroxidase is temporally expressed in germinating cucumber seeds (Fig. 1). A single protein band, corresponding to PRX 1 isoenzyme, was firstly detected in extract of the seedlings 6 d after imbibition. The immunoblot also clearly demonstrated that as the seedlings underwent further development PRX 1 specific signal increased with seedling age to reach maximum intensity 11 d after imbibition.

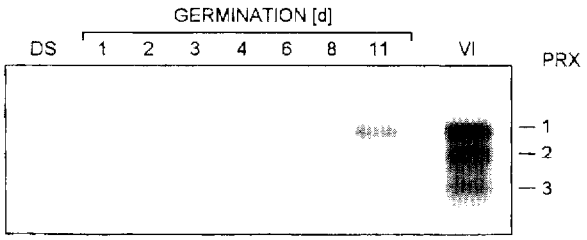

Fig. 1. Western blot analysis of the stress-related anionic peroxidase expression in germinating cucumber seeds. Immunoblot of total protein extracts after separation by native-PAGE (10 %) gel. Lanes labeled as *DS* and *VI* refer to dry seeds or virus-infected (TNV) cotyledons, respectively. 25 μg of total protein was loaded per lane of the gel.

Since the appearance of the PRX 1 immunoreactive signal coincided with the period when the seedlings started to be photosynthetically active, it would be interesting to examine its precise localization using tissue printing technique.

Longitudinal sections of 4-, 6- and 11-d-old cucumber seedlings were pressed gently and evenly onto nitrocellulose membrane. Staining for protein revealed that protein was distributed essentially homogeneously over the different tissues (Fig. 2*a,b,c*). In contrast, from tissue printing evidence, PRX 1 appears to be differentially expressed in various tissues in the course of seedling development.

Immunoscreening of the print blots with antiserum showed that PRX 1 was barely detectable mainly in roots of 4-d-old seedlings (Fig. 2*d*). The absence of the corresponding signal in the PAGE immunoblot may be explained by the fact that tissue print immunoblots were overdeveloped. After 6 d of germination, immunoreactive PRX 1 was again present prominently in root tissue, but it was diffusible distributed throughout the system (Fig. 2*e*). In contrast, in 11-d-old seedlings two kind of immunoreactive signals were observed. Firstly, PRX 1 specific signal was found to be concentrated mainly in the transition zone between roots and stem (Fig. 2*f,g*). On the other hand, unexpectedly the same PRX 1 specific signal was present in cotyledons, particularly in the lower epidermis at the position of the midrib (Fig. 2*f,h*). The identity of both signals detected in different plant tissues was

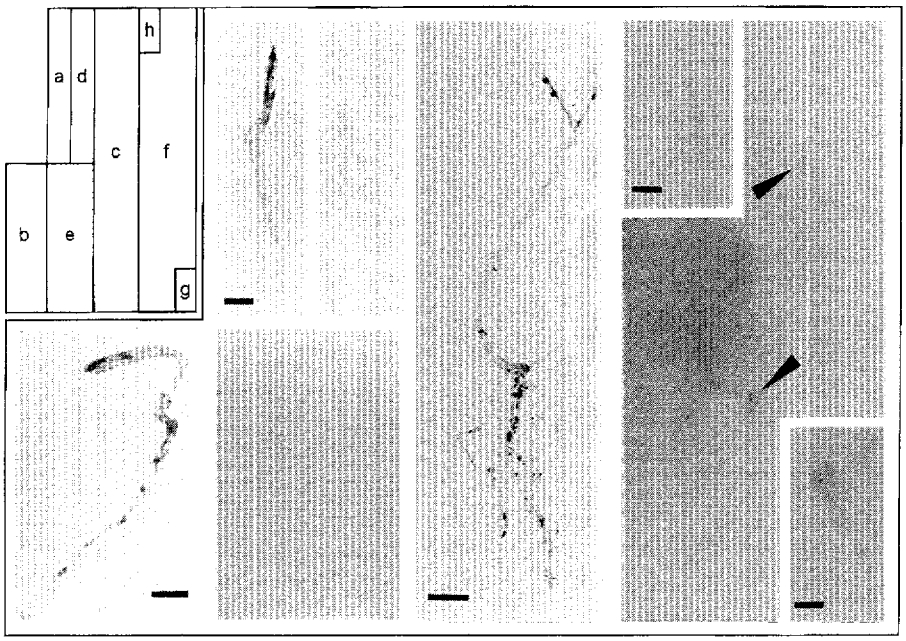

Fig. 2. Tissue prints: *a,d* - 4-d-old cucumber seedling (× 45); *b,e* - 6-d-old cucumber seedling (× 30); *c,f* - 11-d-old cucumber seedling (× 20). Inserts *g,h* represent a higher magnification (× 40) of the areas marked by *arrowheads* in part *f*. Prints were stained for total proteins with Amido black (*a,b,c*) or incubated with the mixed antiserum against PRX 1, 2, 3 (*d,e,f,g,h*). *Arrowheads* indicate the concentrated immunospecific signals. *Bars* - 100 μm.

confirmed by PAGE immunoblot because there was not other protein band cross-reacted with the antiserum.

Although it has been demonstrated that the stress-related anionic peroxidase, comprising of three molecular forms, was strongly induced and accumulated in the extracellular space of TNV-infected cucumber cotyledons (Repka and Slováková 1994), a large body of evidence indicates the possible dual functionality of particular

peroxidase isoenzymes. For example, Repka and Jung (1995) reported the expression of PRX 3 isoenzyme in pistil tissue of cucumber female flowers. Also the results presented in this work suggest that the expression of PRX 1 is rather development- than the pathogenesis-related. This suggestion is strongly supported by two independent findings. Firstly, the germination of seedlings was performed under aseptic conditions. Secondly, the presence of PRX 1 in the roots of germinating seedlings, young as well as in old plants (data not shown), suggest that the expression of this isoenzyme is related to root differentiation. Based on its extracellular localization (Repka *et al.* 1993) it seems likely that PRX 1 participates in the lignification of xylem vessels. The concentration of the PRX 1 immunoreactive signal just in the transition zone between roots and shoot also supports this statement. Such a location might serve as a lignification centre from which both basipetally and acropetally lignification progresses. To corroborate this theory, however, further experiments dealing the histochemical localization of lignin components are needed.

The results presented here show that PRX 1 immunoreactive signal was localized in the lower epidermis of growing cotyledons. The epidermis plays a crucial role in protection of the plant against biotic and abiotic stresses. Many structural biopolymers including lignin, suberin, cutin and extensin may be formed as protective barriers (Fry 1986, Bowles 1990). It may be possible that PRX 1 is involved in the deposition of these biopolymers in the epidermis and in the cell walls surrounding substomatal cavities. In this context, Casal *et al.* (1994) reported that changes in extracellular peroxidase activity correlated in time and localization with changes in lignin content in etiolated *Vicia faba* epicotyls.

The presence of PRX 1 in the aerial plant organs might also be viewed in relation to the proposed role of epidermis in determination of growth rates (Kutschera and Briggs 1987). Impregnation of cell walls with lignin-like materials resulting from the peroxidase-assisted cross-linking of different phenolic compounds reduces the capacity of cells to expand (Taiz 1984, Fry 1986). In this context, it is interesting to note, that none stress-related peroxidase activity was detected in 14-d-old cucumber cotyledons in whose the growth was naturally arrested. The stress-related peroxidase, including PRX 1, appeared again in very old or TNV-infected cotyledons (Repka *et al.* 1993). In order to assess the actual physiological role(s) of PRX 1 isoenzyme in the course of germination programme, its precise immunohistological localization will be needed. This research is in progress.

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