BIOLOGIA PLANTARUM 38 (4): 571-583, 1996

Distribution of the stress-related anionic peroxidases in different cucumber organs

V. REPKA and I. FISCHEROVÁ

Laboratory of Molecular Biology and Virology, Complex Research Institute of Viticulture and Enology, Matúškova 25, SK-833 11 Bratislava, Slovakia

Abstract

The distribution of the stress-related anionic peroxidase (srPRX) activity was investigated in various organs of cucumber (*Cucumis sativus* L. cv. Laura) during their development by activity staining and immunoblotting. In shoots, including cotyledon, leaf, stem and tendril, three stress-related peroxidase isoenzymes were present, particularly in old ones. The PRX1 was the only srPRX isoenzyme found in both young and old roots. As fruits became mature, srPRX activity increased dramatically and was particularly enriched in the external parts of the fruit. The PRX1 isoenzyme was highly accumulated in the course of seed germination, while the absence of other two srPRX isoenzymes (PRX2 and PRX3) was recorded. The possible function of the srPRX is discussed, with respect to this spatio-temporal distribution.

Additional key words: Cucumis, electrophoresis, fruits, immunological study, seed germination, tobacco necrosis virus, vegetative tissues, Western blotting.

Introduction

Biological stress as a result of pathogen infection causes extensive changes in synthesis of proteins that are related to defense responses. Cucumber stress-related peroxidase (srPRX) is one of the most prominent soluble proteins present in the extracellular space of virus-infected cucumber cotyledons (Repka *et al.* 1993). Upon gel electrophoresis of intercellular fluid (ICF) extracted from this organ under native conditions srPRX is visualized as a fast-moving anionic group of three enzymatically active bands PRX 1,2 and 3 (Repka and Slováková 1994). Beside different stress

Received 12 February 1996, *accepted* 9 May 1996.

Abbreviations: AFL - average fruit length; BSA - bovine serum albumine; DR - defence-related proteins; EDTA - ethylenediamintetraacetic acid; ICF - intercellular fluid; NC - nitrocellulose; PAGE - polyacrylamide gel electrophoresis; PVP - polyvinylpyrrolidone; srPRX - stress-related peroxidase; TNV - tobacco necrosis virus.

Acknowledgements: The authors thank Mgr. Katarína Čanigová for her excellent technical assistance and Dr. Ladislav Tamás for his help with densitometry and computer analysis.

conditions (Repka and Vanek 1993) we have already reported that srPRX activity is present in different parts of healthy cucumber plant (*e.g.* male and female flowers, Repka and Jung 1995). Although this observation is fully consistent with the fact that many peroxidase isoenzymes are organ-specific and their expression is usually under developmental control (Gaspar *et al.* 1982, Greppin *et al.* 1986), there is no substantial information available about the function of the stress-related peroxidase isoenzymes during healthy plant development.

In this context, it is also interesting that several recent studies revealed the presence of different defence-related (DR) proteins in relation to specific developmental stages or organs (Felix and Meins 1986, Lotan *et al.* 1989, Memelink *et al.* 1990, Ori *et al.* 1990, Grosset *et al.* 1990, Tiré *et al.* 1994). To extend our previous findings concerning the differential expression of the srPRX in cucumber flowers we determined spatial and temporal expression of the srPRX set in other organs of cucumber plant. In this report, we describe the results of immunoblotting experiments using antibodies directed against srPRX proteins extracted and purified from TNV-infected cucumber cotyledons (Repka and Slováková 1994).

Material and methods

Plants: Cucumber (*Cucumis sativus* L. cv. Laura) seeds were surface sterilized with 0.8 % Domestos (*Unilever Ltd.*, Bratislava, Slovakia), washed at least three times with sterile distilled water and transferred under aseptic conditions into the sterile Petri dishes containing filter paper discs soaked with half-strength MS medium (Murashige and Skoog 1962) lacking phytohormones. The seeds were placed into cultivation chamber and allowed to germinate at 22 °C for 11 d at a 12 h photoperiod.

Plants were grown in a greenhouse under controlled conditions (Repka and Slováková 1994). Samples *a*) cotyledons and leaves from 7-d and 2-weeks-old seedlings, *b*) roots from 1- to 10-weeks-old plants, *c*) stems and tendrils from 12-weeks-old plants, *d*) immature fruits from 2- or 6-weeks-old plants and mature fruits from 12-weeks-old plants were collected from at least five plants.

Inoculation of plants: Fully expanded cotyledons (7-d-old) were abrased and inoculated with a suspension of TNV as described previously (Repka and Slováková 1994). Control plants were treated similarly with virus isolation buffer. Plants were then incubated under standard conditions. The cotyledons bearing necrotic lesions were harvested 7 d after inoculation and stored at -20 °C or processed immediately.

Preparation of plant extracts: Plants were harvested, divided to individual organs and ground with pestle and mortar 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). Homogenate was centrifuged at 15 000 *g* for 20 min at 4 °C. The clear supernatant obtained after centrifugation was concentrated on *Microcon-3* microconcentrator (*Amicon GmbH.*, Witten, Germany) and stored frozen at -20 °C.

Sample analysis: Contents of total proteins in homogenates were determined colorimetrically using the Bradford (1976) staining procedure, using BSA as standard. Peroxidase activities were determined as described (Repka and Slováková 1994) in 50 mM sodium acetate buffer and enzyme activity was expressed as change in the absorbance at 470 nm [ΔA_{470} mg^{-1}(protein) s^{-1}].

Analytical PAGE: Proteins were separated by non-denaturing PAGE using a 4 % stacking gel and 10 % separation gel. Each lane was loaded with equal amounts of protein and bromphenol blue as marker. Peroxidase isoenzymes on gels were visualized basically according to Repka and Jung (1995) with the exception that 3-amino-9-ethylcarbazole (0.04 %) was used instead of 3,3'-diaminobenzidine.

Western blotting: Proteins separated on PAGE gels were blotted onto *NC-membrane* (*Protran BA-85*, 0.45 μm, *Schleicher and Schuell*, Dassell, Germany) and probed with specific sera raised against purified srPRX (Repka and Slováková 1994). In order to obtain a complex pattern of srPRX expression, a mixture of cucumber anti-PRX 1,2 and 3 antibodies was employed.

Densitometric analysis: Protein bands on the dried activity stained gels and membranes were analyzed in a *UVP* computerized densitometer *GDS 5000* (*UVP Products*, Cambridge, UK) equipped with a powerful gel analysis and documentation software.

Results

Stress-related peroxidase activity in vegetative organs: There is no quantitative difference in the total peroxidase activity between the both young and old cotyledons

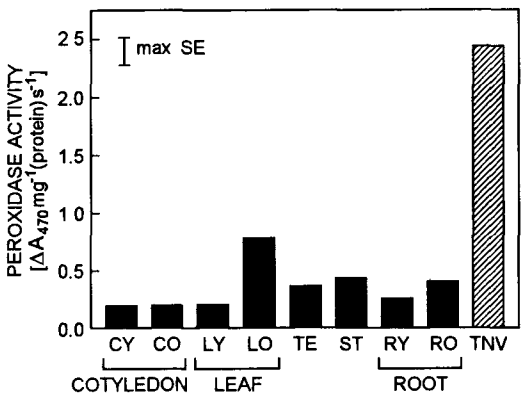

Fig. 1. The total soluble peroxidase activity in extracts from various vegetative organs and from TNV-infected cucumber cotyledons (means of three replicates and maximal S.E. (*vertical bar*); Y - young, O - old, TNV- tobacco necrosis virus infected cotyledons).

(Fig. 1). An inverse pattern was observed for leaves where the total soluble peroxidase activity in old tissues represented at about fourfold increase of that in young leaves. About equal increase of total peroxidase activity was observed in stem and tendrils. The difference in accumulation of the total soluble peroxidase activity between young and old roots copy the pattern typical for leaves, although in a lesser extent.

Extracts from corresponding vegetative organs were examined for the presence of the srPRX isoenzymes by native PAGE for acidic proteins. No srPRX electrophoretic forms could be detected in young cotyledon and young leaf extracts but only in old ones (Fig. 2*a*). The previously reported srPRX isoenzymes were found in both old organs and the intensity of the three acidic peroxidase activities was high when compared to TNV-infected cotyledons. Stem and tendrils also accumulated three respective srPRX isoenzymes although in somewhat lower rate than in old cotyledons or leaves. The PRX1 was the only srPRX isoenzyme expressed in both young and old root extracts.

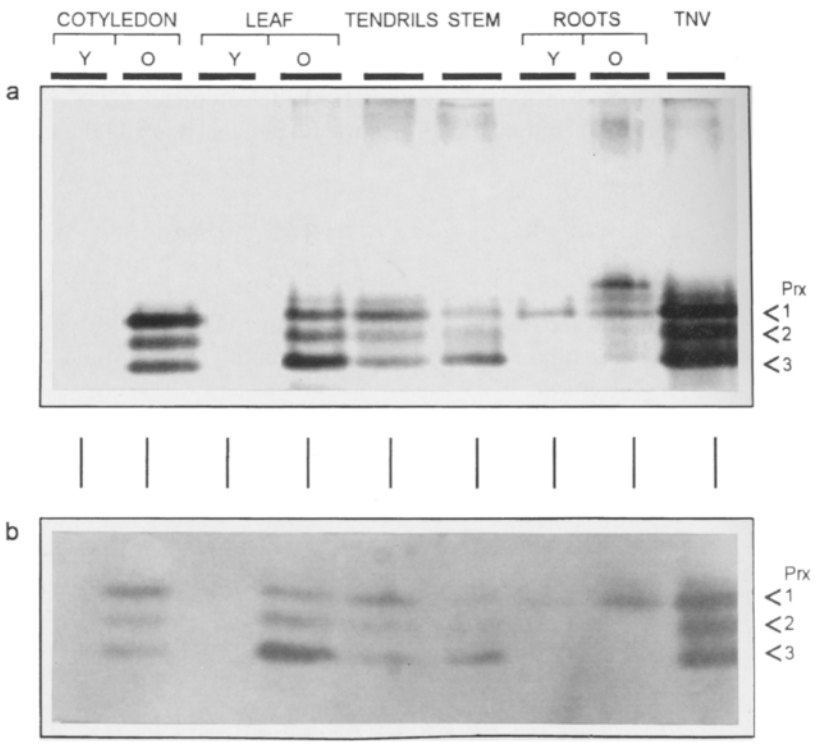

Fig. 2. Comparative 10 % polyacrylamide gel electrophoresis under native conditions. Proteins were extracted from organs as indicated and each lane was loaded with 50 μg of proteins. The gel was stained for isoperoxidase pattern (*a*) or transferred to *NC*-membrane and immunodecorated with specific antiserum raised against purified cucumber peroxidase (*b*) (Y - young, O - old, TNV - tobacco necrosis virus infected cotyledons).

The Western blotting analysis of the particular extracts (Fig. 2*b*) confirmed the expression patterns of the srPRX observed on activity stained gel. Immunodetection with highly specific antiserum also more finely revealed the difference in the expression rate among individual srPRX isoenzymes in particular organ as well as among the respective organs.

Densitometry and computer analysis of the immunoblotting experiment precisely documented a diversity in distribution of the particular srPRX isoenzymes present in extracts from various vegetative organs (Fig. 3). Besides mainly quantitative differences, a typical pattern of expression was characteristic for each type of the examined organ.

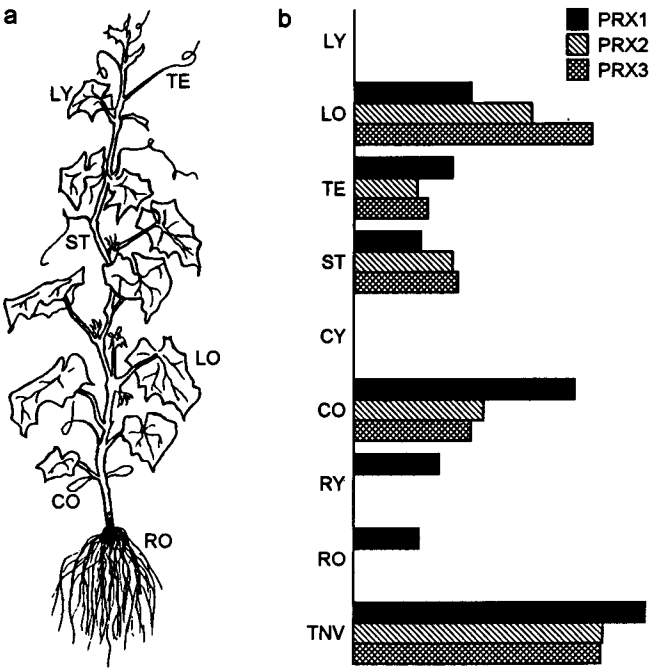

Fig. 3. Computer-assisted distribution analysis of individual srPRX isoenzymes in different vegetative organs (*a*, *b*). The *bar heights* are proportional to the areas of the peroxidase protein bands in Fig. 2*a* resulting from densitometric scanning (LY - leaf young, LO - leaf old, TE - tendrils, ST - stem, CY - cotyledon young, CO - cotyledon old, RY - root young, RO - root old, TNV - tobacco necrosis virus infected cotyledons).

Stress-related peroxidase activity in fruits: Ovary extracts from 2- and 6-week-old (immature) and 12-week-old (mature) cucumber fruits were analysed and accumulation of the total soluble peroxidases showed a decreasing pattern towards the fruit maturation (Fig. 4*a*). There was a significant change when the same extracts were subjected to native PAGE for acidic proteins. While the highest difference in the total soluble peroxidase activity was recorded between 2- and 6-week-old fruits, no comparable diversity was observed on activity stained gel (Fig. 4*b*). Except two

slow-moving anionic peroxidase isoenzymes, visible as the smears on the top of the gel, there is neither qualitative nor quantitative difference between the two stages.

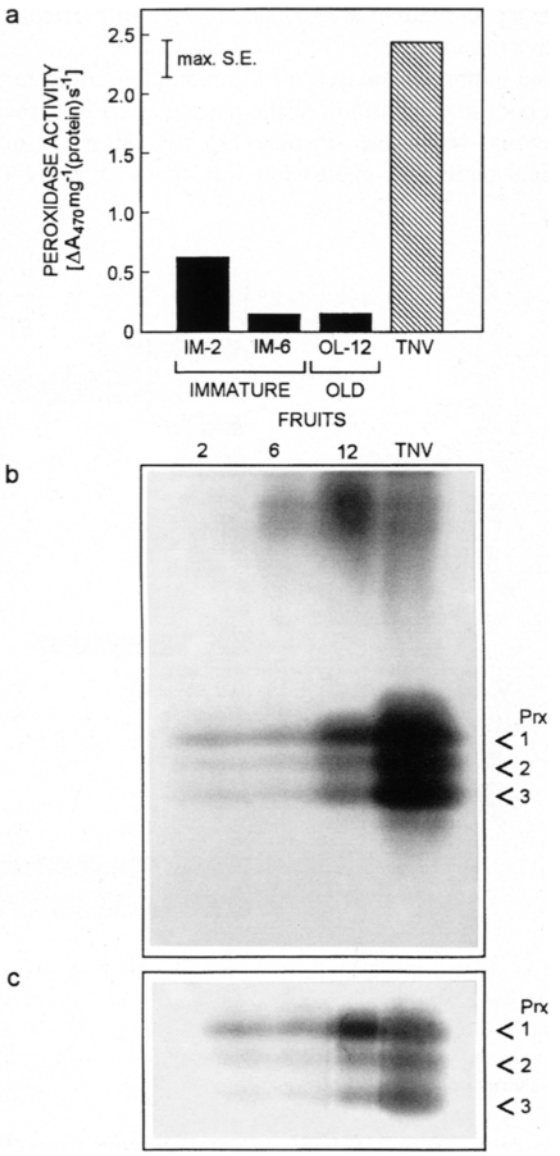

Fig. 4. Peroxidase activity in fruits. Immature fruits (2- and 6-week-old, IM-2 and IM-6, respectively) and mature fruit (12-week-old, OL-12) extracts were made. The extracts were assayed for the total soluble peroxidase activity (*a*). Values are means of three separate measurements. Aliquots of 50 μg and 100 μg were subjected to native PAGE and stained for peroxidase activity (*b*) or immunodecorated with specific antiserum (*c*), respectively. Numbers at top of Fig. 4*b* denote 2-, 6- and 12-week-old fruits.

Much higher rate of accumulation of the srPRX was observed in mature (12-week -old) fruits. This pattern of accumulation was verified by Western blotting (Fig. 4*c*). Moreover, the PRX1 activity was the major signal in all three stages and it increased with fruit aging. As revealed by densitometry, the distribution of individual srPRX isoenzymes did not change dramatically in the course of fruit maturation (Fig. 5).

The extracts from various parts (epidermis, subepidermis, fruit wall, placenta and immature seeds) of mature fruits of 12 weeks were tested similarly as above. While the highest rate of accumulation was detected in epidermis, striking gradient in decrease of the total soluble peroxidase activity towards the placenta tissue was recorded. Peroxidase activity slightly increased again in immature seeds.

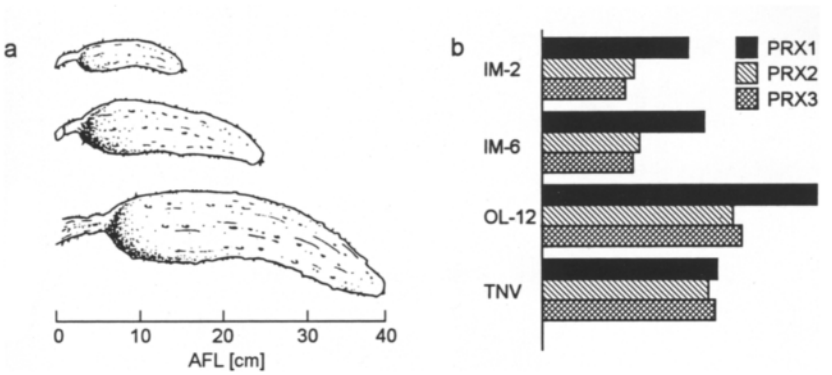

Fig. 5. Distribution analysis of individual srPRX isoenzymes in fruits of different age (*a*). *Bar heights* are proportional to the areas of the peroxidase protein bands in Fig. 4*b* resulting from densitometric scanning and computation (*b*) (IM-2, IM-6 and OL-12 refer to the 2-,6- and 12-week-old fruits).

Activity staining of anionic peroxidases on the PAGE gel revealed the presence of all three previously identified srPRX isoenzymes except in immature seeds (Fig. 6*b*). The same pattern of the srPRX isoenzymes expression was detected by Western blotting of the comparable gel (Fig. 6*c*).

Distribution analysis estimated by densitometry (Fig. 7) clearly demonstrated that in mature fruits, the srPRX activity was higher in the external parts (epidermis, subepidermis and fruit wall) than in the internal parts (placenta, immature seeds) of the fruit.

Stress-related peroxidase activity in seeds: There was not striking difference in accumulation of the total peroxidase between the dry seeds and seeds germinating for 1 and 2 d. Starting from the third day of germination, a marked accumulation of the total soluble peroxidase activity was observed. The maximum rate of accumulation was reached on the 8th day of germination, followed by decrease of the intensity as germination progressed.

No difference in the srPRX levels could be detected on the activity stained gel (Fig. 8*b*) among seeds germinating for 1 - 3 d. Later on, however, there was a marked increase of the srPRX expression starting the 4th day after sowing. From the three

srPRX isoenzymes, the PRX1 was the only isoenzyme markedly accumulated in the course of seed germination. There was a minor faint PRX2 signal detected 11th day of germination.

The Western blotting analysis positively confirmed the temporal mode of the PRX1 isoenzyme accumulation during germination (Fig. 8*c*), although the barely detectable isoenzyme PRX2 on activity stained gel was just at the limit of immunoblot analysis. Fig. 9 shows a distributions pattern of the Prx 1 isoenzyme in the progression of germination as revealed by densitometry. It is noteworthy, that the rate of accumulation on the 11th day of germination represents at about the twofold increase than that in TNV-infected cotyledons.

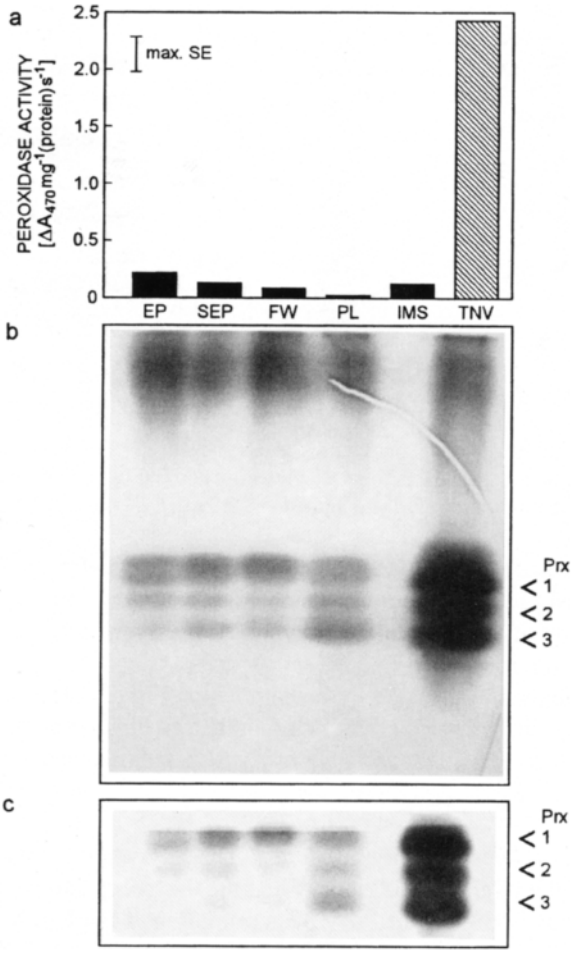

Fig. 6. The total peroxidase activity (*a*), native PAGE activity staining (*b*) and Western blotting analysis (*c*) of the tissue specific patterns in mature fruit. For activity staining and immunoblotting each lane was loaded with 50 and 100 μg of total proteins, respectively (EP - epidermis, SEP - subepidermis, FW - fruit wall, PL - placenta, IMS - immature seeds, TNV - tobacco necrosis virus infected cotyledons).

Discussion

As revealed by activity staining and comparable immunoblot, all three srPRX isoenzymes were present in cucumber shoots (cotyledons, leaves, stem and tendrils). It was also documented that expression of srPRX, at least in cotyledon and leaf, is strictly ontogenetically regulated since it accumulates only in old tissues. In spite of these facts, the expression of the srPRX in old cucumber tissues is probably related to leaf senescence or might be related to a decrease in growth. In respect to both, stem and tendrils, it is intriguing what is the physiological role(s) of the srPRX in these organs, since whole stems and tendrils from whole plant were taken under investigation. Based on its distribution, as revealed by computer densitometry, there was evident a marked diversity in expression of particular isoenzymes (especially PRX 1 and 3) between the two organs.

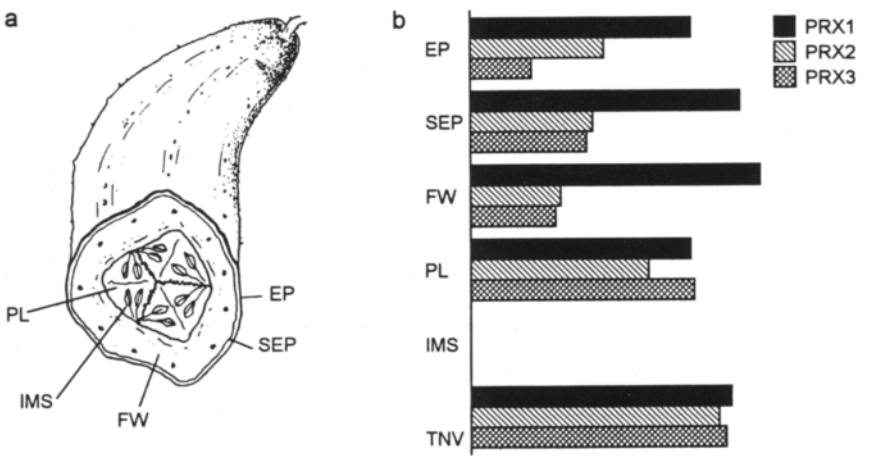

Fig. 7. Tissue-specific distribution of individual srPRX isoenzymes in mature cucumber fruit (*a*). The *bar heights* (*b*) are proportional to the areas of peroxidase protein bands in Fig. 6*b* resulting from densitometric scanning and computer analysis (EP - epidermis, SEP - subepidermis, FW - fruit wall, PL - placenta, IMS - immature seeds, TNV - tobacco necrosis virus infected cotyledons).

In this context, a different pattern of the srPRX expression was observed for roots. Immunoblotting positively revealed that practically there is no difference, except quantitative one, between the both young and old roots. In addition, the PRX1 was the only srPRX isoenzyme accumulated in roots, although extract from the old roots stained for an anionic peroxidase activity contained at least four additional isoenzymes when compared with young one. Due to its low-level expression it seems likely, that PRX1 is instrumental in the lignification of xylem vessels of roots. Whether or not it is the case, further experiments must be done regarding the immunohistochemical localization of this isoenzyme in the organ.

The srPRX was found also in the course of fruit development. It is interesting, however, that while in immature fruits the level of expression of srPRX remained

relatively constant, it was highly increased as the fruit became older. Similar situation has been observed for another PR-protein (chitosanase), where the highest overall amount of this activity in cucumber was found in fruits. Separation of the mature fruit into the individual tissues revealed that srPRX activity is contributed mainly to the external parts of the fruit underlying its putative defense role. An endogenous role, although of undetermined origin, is also possible. An interesting feature was the absence of the srPRX in immature seeds, but this situation is not quite surprising, since it was typical for the other DR-products (*e.g.* chitosanase, Ouakfaoui and Asselin 1992).

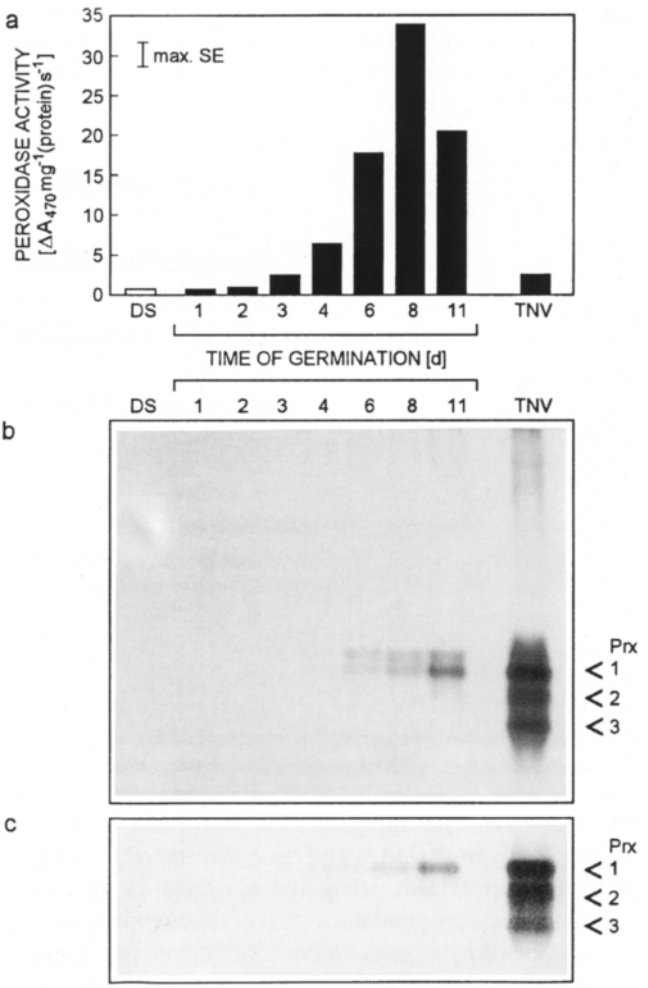

Fig. 8. Peroxidase activity in seeds. The total peroxidase activity in extracts from dry seeds and from seeds germinating for 1 - 11 d (*a*) [means and maximal S.E. (*vertical bar*) of three replicates]. Comparative 10 % native PAGE. Gels were either activity stained (*b*) or immunodecorated (*c*) using the antiserum against purified cucumber peroxidase. Each lane on the gel was loaded with 50 and 100 μg of total proteins, respectively.

A specific pattern of the srPRX expression was found in germinating cucumber seeds. Firstly, the PRX1 was the only srPRX isoenzyme accumulated in germinating seeds starting from the 4th day after sowing. Secondly, this pattern coincides with the temporal mode of accumulation reported for another oxidoreductase - lipoxygenase (Matsui *et al.* 1992). Its activity increased rapidly from 3 d after imbibition of light grown cucumber seedlings. Recently, it was also reported that barley aleurone layer highly accumulates β-1,3-glucanase (Leah *et al.* 1991) and chitinase (Jacobsen *et al.* 1990) during germination. The work of Ouakfaoui and Asselin (1992) confirmed that the cucumber seed chlorenchyma tissue is so highly enriched in chitosanase activity as germination progressed. At the present, the potential function(s) resulting from a massive accumulation of various PR-proteins including srPRX remains unknown.

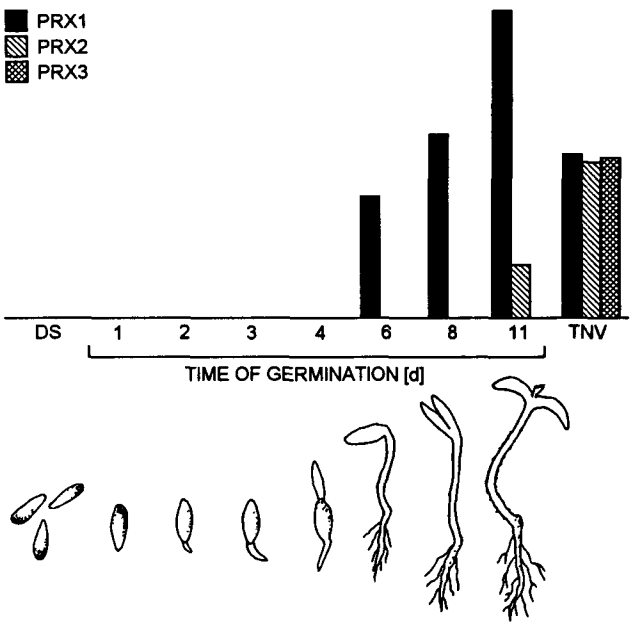

Fig. 9. Distribution of individual srPRX isoenzymes in germinating cucumber seeds. *Bar heights* are proportional to the areas of the peroxidase protein bands in Fig. 8*b* resulting from densitometric scanning and computer analysis (DS - dry seeds, TNV- tobacco necrosis virus infected cotyledons).

In respect to the accumulation of the srPRX in the course of seed germination, there are several possible explanations, none of which are mutually exclusive. Firstly, the increase in srPRX level suggests that this activity could possible serve a putative antimicrobial function. Secondly, since the increased accumulation of the srPRX coincided with the period when seedling cotyledons start to be photosynthetically active (6-d after sowing), it is reasonable to assume that it may be related to light. Additionally, in this context, it was very well demonstrated that phytochrome status influences extracellular peroxidase activity in etiolated (Kim *et al.* 1989) and light-grown (Casal *et al.* 1990) seedlings of different species. Moreover, Casal *et al.* (1994) demonstrated that light induced changes in

extracellular peroxidase activity correlated in time and localization with changes in lignin content in the cortical parenchyma of *Vicia faba* epicotyls. To finally assess the possible role of light on the srPRX accumulation in seeds, a set of extensive experiments must be performed regarding the germination of seedlings under various light regimes.

In conclusion, these results show that srPRX expression in cucumber is variable and strictly depends on a spatio-temporal stages of plant development.

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