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Light-induced changes in expression of pathogenesis-related anionic peroxidase in cucumber seedlings

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Abstract

The effect of irradiance on the expression of the *Cucumis sativus* pathogenesis-related (srPRX) anionic peroxidase was studied in germinating seeds at the period when seedlings start to be photosynthetically active. The diversity in the expression patterns of srPRX was observed in both dark- and light-grown seedlings using activity staining and immunoblotting: beside the three srPRX isoenzymes also other three, serologically unrelated, peroxidase isoforms were accumulated in dark-grown seedlings and one in light-grown seedlings. Furthermore, in light-grown seedlings, it was observed a marked difference in the expression of particular srPRX isoenzymes at higher irradiance (up to 260 W m^{-2}, 400 - 700 nm) in comparison to low irradiance. By tissue printing on nitrocellulose paper it was demonstrated that irradiance during germination induced changes in the temporal and spatial distribution of the srPRX.

Additional key words: Cucumis, electrophoresis, immunoblotting, localization, tissue printing.

Introduction

Pathogenesis-related (PR) proteins were originally defined as a group of acidic-type proteins that accumulate in the extracellular space of tobacco leaves hypersensitively reacting to virus infection (Van Loon and Van Kammen 1970, Parent and Asselin

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Abbreviations: ABA - abscisic acid, BSA - bovine serum albumine, EDTA - ethylenediaminetetraacetic acid, DAB - 3,3-diaminobenzidine, 1/2 MS - half strengh Murashige and Skoog medium, ICF - intercellular fluid, LOX - lipoxygenase, NC - nitrocellulose, PAGE - polyacrylamide gel electrophoresis, PAR - photosynthetically active radiation, PR - pathogenesis-related, PRX - peroxidase, PVP - polyvinylpyrrolidone, srPRX - stress-related peroxidase, TNV - tobacco necrosis virus.

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1984, Bol *et al.* 1990). Beside pathogen infection, several environmental and hormonal signals are known to control the expression of PR-genes. These signals include ethylene, wounding, abscisic acid (ABA), salt stress, UV radiation, auxin and cytokinin (Brederode *et al.* 1991, Cutt and Klessig 1992, Kononowicz *et al.* 1993, Singh *et al.* 1987). Additionally, there are several lanes of evidence which suggest that the light is another, probably very important, factor involved in the control of PR-gene expression. Light-dependent induction of acidic-type PR-proteins has been previously noted (Asselin *et al.* 1985, Lotan and Fluhr 1990 a,b). More recently, it has been shown that the light requirement is not an inherent character of the gene, but apparently influences the signal transduction pathway (Eyal *et al.* 1992).

In cucumber we have shown earlier that pathogenesis-, or a more broadly, stress-related anionic peroxidase (srPRX) was highly accumulated in seedlings just at the period when these start to be photosynthetically active (Repka and Fischerová 1996). Although this stress-related peroxidase consists of three serologically related molecular forms called PRX 1, 2 and 3 (Repka and Slováková 1994), using the specific antiserum it has been shown that the PRX 1 was the only isoenzyme accumulated in germinating seeds (Repka and Fischerová 1996). Furthermore, our recent study on the *in situ* localization of the PRX 1 isoenzyme on tissue-print immunoblots confirmed an accumulation of this product in photosynthetically active tissues of cotyledons (Repka *et al.* 1997).

Since there appears to be a relationship between the light and srPRX expression in photosynthetically active cucumber seedlings, the aim of this study was to reach a better understanding of the functional ground for this type of accumulation.

Materials 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 moistened with 1/2 MS medium (Murashige and Skoog 1962) lacking phytohormones. The Petri dishes were placed into a ventilated cultivation chamber [temperature 22 °C, relative humidity 60 %, combination of *Cool White* and *Grolux* fluorescent lamps (*Sylvania*)] and allowed to germinate under various light regimes. In the first group of experiments the seedlings were germinated for 11 d at: *a*) constant darkness, *b*) 12-h photoperiod, irradiance of 130 W m^{-2} PAR at the surface of the Petri dishes, *c*) continuous light, irradiance of 130 W m^{-2}. In the second group of experiments the seeds were germinated for 6, 8 or 11 d under various irradiances: 65, 130, 195, 260 W m^{-2}. Three Petri dishes each containing 25 seeds were taken per experiment. Cotyledons of *ca.* 7-d-old cucumber seedlings were dusted with carborundum and inoculated with partially purified suspension of TNV virus. The isolation of intercellular fluid was performed according to the published protocol (Repka and Slováková 1994).

Preparation of plant extracts: After the treatment, seedlings were harvested and ground with pestle and mortar in appropriate volumes of *Trisepac* buffer (50 mM Tris-HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA, 0.2 % insoluble PVP, 6 mM ascorbic acid and 0.1 % cysteine). Homogenates were centrifuged at 15 000 *g* for 20 min at 4 °C. The clear supernatants obtained after centrifugation were further concentrated using *Microcon-3* microconcentrators (*Amicon GmbH.*, Witten, Germany). The samples were stored at -20 °C until future use. All these steps were done under dim green light.

Sample analysis: The protein concentration in the extracts was determined by the method of Bradford (1976), using BSA as standard. Peroxidase activity was determined in the concentrated plant extracts following the protocol described (Repka and Slováková 1994) in 50 mM sodium acetate buffer (pH 5.2). The enzyme activity was expressed as a change in the absorbance at 470 nm [ΔA_{470} mg^{-1}(protein) s^{-1}].

Analytical PAGE and staining of peroxidase isoenzymes: Proteins were separated by non-denaturing PAGE using a 4 % stacking gel and 10 % separating gel. Each lane of the gel was loaded with equal amounts of protein and bromphenol blue as marker. Peroxidase isoenzymes were visualized either with starch/KI reagent of Olson and Varner (1993) following the protocol of Repka and Fischerová (1997) or using 0.03 % DAB according to Repka and Jung (1995).

Western blotting: After the separation was completed, proteins were transferred onto NC-membrane (*Protran BA-85*, 0.45 mm, *Schleicher and Schuell*, Dassell, Germany) at 4 °C for 20 h at 50 mA using 0.7 % acetic acid as a transfer buffer. Following the transfer, the membrane was baked at 80 °C for at least 8 h to inactivate endogenous peroxidases. After blocking the membrane with 5 % *Blotto* (nonfat dry milk), the Western blot was probed with specific antisera raised against purified srPRX and further processed according to Repka and Slováková (1994). To obtain a complex pattern of srPRX expression, a mixture of cucumber anti-PRX 1, 2 and 3 antibodies was employed.

Tissue printing: For tissue printing, a modification of standard procedure (Cassab and Varner 1987) was used. Longitudinal fresh hand-sections were pressed gently and evenly for 20 s onto 0.45 mm *Protran* nitrocellulose paper (*Schleicher and Schuell*, Dassell, Germany) where the pretreatment step with 0.2 M $CaCl_2$ was omitted. At least two replicate prints were made with the same age materials. Following the printing, the membrane was baked and immunoprocessed according our standard Western blotting protocol (Repka and Slováková 1994). Controls included no antibody (blocking buffer substituted) or secondary antibody only. The other two sets of tissue prints were stained for total peroxidase activity using 0.03 % DAB (*Amresco*, Solon, USA) and for total proteins using 0.1 % solution of *Amido* black. Tissue prints were photographed with *Kodak Gold* 100 ASA (*Eastman Kodak*, Rochester, USA) using *Leica* stereomicroscope *MZ12* (*Leica*, Heerbrugg, Switzerland) equipped with the Trinocular video/phototube. Black and white prints were made using *Agfa* multigrade paper.

Results

Effect of the light regime on the total soluble peroxidase activity and expression of the srPRX: Since no srPRX accumulation was observed up to 3rd d of germination 4-d and older seedlings were taken for experiment. Based on enzyme activity measurements, light had a profound effect on the total peroxidase accumulation (Fig. 1). Starting from day 6 peroxidase activity increased dramatically in both dark- and light-treated seedlings, and the highest activity was observed in 11-d-old seedlings. In seedlings grown at continuous light or darkness the activity was 4-fold or 2-fold, respectively, higher that in those grown at 12-h photoperiod.

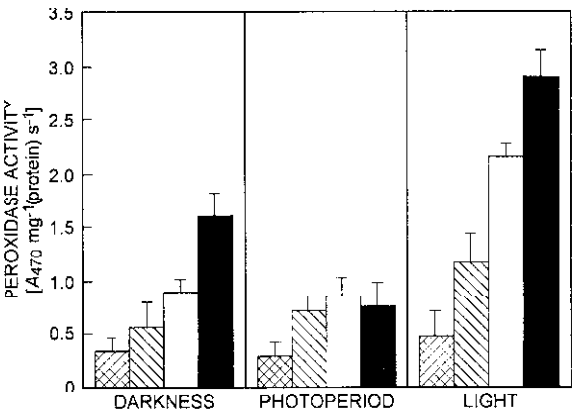

Fig. 1. Time-course of total soluble peroxidase activity in extracts from cucumber seedlings grown 4, 6, 8 and 11 d (*crossed, hatched, open* and *full columns*, respectively) under continuous darkness, 12-h photoperiod or continuous irradiance (130 W m^{-2}) (means of three separate measurements, bars indicate S.E.).

In the seedlings grown at 12-h photoperiod, the PRX 1 was the only srPRX isoenzyme accumulating from 6th day of germination (Fig. 2*A*). The same time course pattern of accumulation was observed for PRX 1 isoenzyme in dark-grown seedlings with the exception that isoenzymes PRX 2 and 3 were highly accumulated on the 11th day of germination. In contrast, all three srPRX isoenzymes were accumulated already in 6 d old seedlings grown under continuous light. It is important to note that there was a set of anionic peroxidase isoenzymes coexpressed together with srPRX ones either in dark- or light-grown seedlings. Beside the PRX 1, 2 and 3 isoenzymes, three other peroxidases termed as PRX 4, 5 and 6 were accumulated in seedlings incubated in darkness. On the other hand, the PRX 6 isoenzyme was present also in seedlings grown under continuous light.

The Western blotting analysis of the particular extracts confirmed the expression patterns for the srPRX isoenzymes observed on activity stained gel (Fig. 2*B*). Immunoblotting with highly specific antiserum failed in the detection of PRX 4, 5 and 6, thus these peroxidases must be serologically unrelated to srPRX.

Effect of different irradiances on the total soluble peroxidase activity and expression of the srPRX: Growth of seedlings for 6 - 11 d at continuous irradiances up to 195 W m^{-2} gradually stimulated the total soluble peroxidase activity, with a maximum reached in 11-d-old seedlings (for 65 and 130 W m^{-2}) or in 8-d-old seedlings (for 195 W m^{-2}, Fig. 3). In contrast, the higher irradiance (260 W m^{-2}) had quite reverse effect and the total peroxidase activity was inhibited when compared to that observed at irradiance 195 W m^{-2}. Although in this case the total soluble peroxidase activity was higher than activity documented either in 65 or 130 W m^{-2}; it gradually decreases as germination progressed.

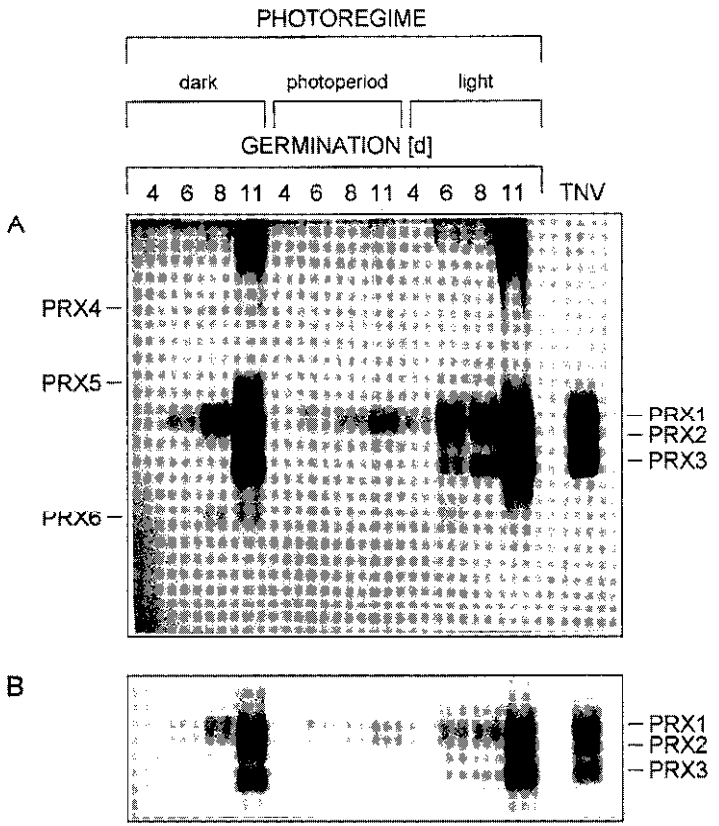

Fig. 2. Gel electrophoretical analysis of time-course of the anionic PRX isoenzymes separated on 10 % PAGE gel under non-denaturing conditions. Proteins were extracted as indicated and each lane of the gel was loaded with 25 μg of proteins. The gel was stained either for isoperoxidase pattern (*A*) or transferred to NC-membrane and immunodetected with specific antiserum raised against purified cucumber srPRX (*B*). Lane denoted as TNV contains 10 μg of the ICF proteins extracted from TNV-infected cucumber cotyledons. DRK - darkness, PHTP - photoperiod, LGT - continuous light

Two different expression patterns were typical for particular srPRX isoenzymes when the corresponding extracts were examined by native PAGE for acidic proteins (Fig. 4*A*). While the expression of srPRX isoenzymes copy the pattern of accumulation of total soluble peroxidase in seedlings treated either with 65 or 130 W m^{-2}, it was not the case for the expression of anionic peroxidases accumulated in seedlings germinated under higher irradiances. Activity stained gel also shows that all three srPRX isoenzymes were highly accumulated in 8-d-old seedlings at irradiance

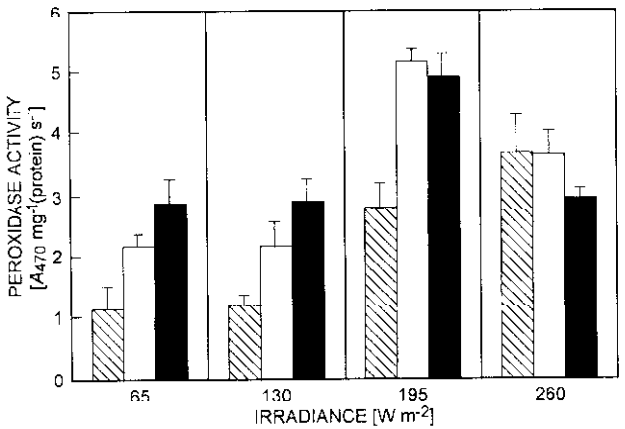

Fig. 3. Total soluble peroxidase activity in extracts from seedlings grown under irradiance of 65, 130, 195 or 260 W m^{-2} for 6 (*hatched columns*), 8 (*open columns*) and 11 (*full columns*) days (means of three separate measurements, bars denote S.E.).

195 W m^{-2} and in a more lesser extent in 11-d-old seedlings. On the other hand, no srPRX isoenzymes accumulation was observed in the extracts from 6- or 8-d-old seedlings under irradiance of 260 W m^{-2}, although all three isoenzymes were expressed in 11-d-old seedlings.

Subsequently, the Western blotting analysis of the corresponding extracts (Fig. 4*B*) confirmed the expression pattern of the srPRX observed on activity stained gel.

Temporal and spatial distribution of the srPRX in etiolated and light-grown seedlings: A series of longitudinal tissue prints were made on nitrocellulose paper from cucumber seedlings germinated under different photoregimes for 6, 8 and 11 d. Staining for total protein revealed that with the except of dark-grown seedlings the protein was distributed essentially homogeneously over the different tissues (Fig. 5, protein column). In etiolated seedlings the protein is mostly found in cotyledons while very low concentration was detected in the root system. The same situation was observed also in 11-d-old seedlings germinated under low irradiance of 130 W m^{-2}.

In contrast, from tissue printing, total PRX appears to be differentially expressed during seedling development under various irradiances (Fig. 5, PRX column). It is evident that there is a progressive increase of total peroxidase in 11-d-old etiolated seedlings. In contrast to staining for total protein, very high content of total PRX was detected in roots of these seedlings. On the other hand, in seedlings grown under low

irradiance, there was a progressive decrease of total PRX activity. Moreover, in seedlings germinated for 8 and 11 d under high irradiance the total PRX activity was concentrated mainly in the roots.

Immunoscreening of the print blots with antiserum raised against purified cucumber srPRX showed that photoperiod and irradiance causes the changes in the spatial and temporal distribution of the srPRX, and a set of morphological and physiological effects on the seedling (Fig. 5, anti-PRX column). No immunoreactive signal was detected in seedlings germinated for 6 d under either of applied treatments. As germination progressed, the spatial pattern of distribution was observed. In 8-d-old seedlings grown under dark or low irradiance the immunopositive signal was almost homogeneously distributed throughout the seedling, while in seedlings grown under higher irradiances the immunoreactive signal started to be concentrated in both cotyledonary tissue and transition zone between the stem and roots.

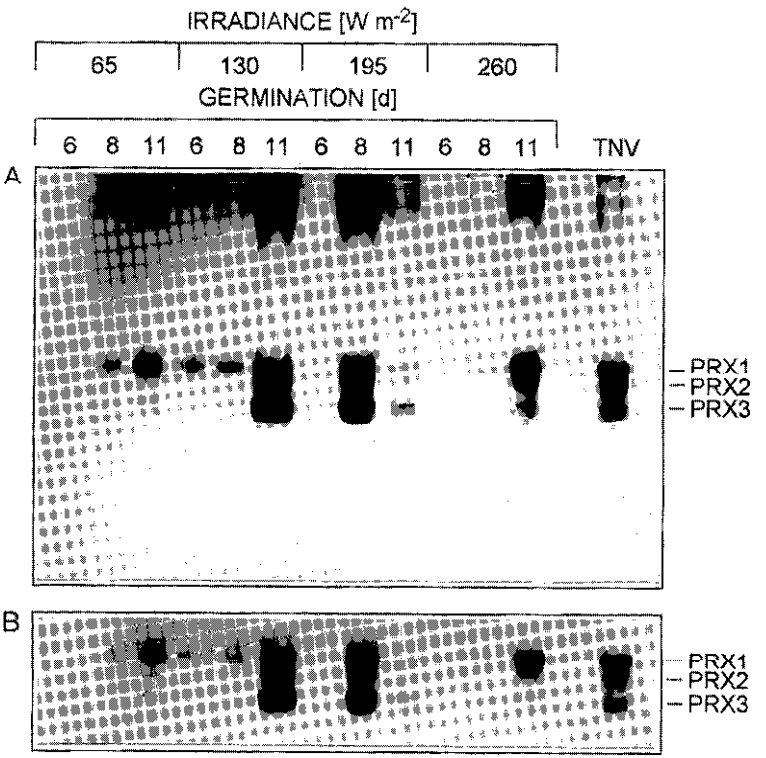

Fig. 4. Electrophoretical analysis of the anionic peroxidase isoenzymes pattern in seedlings grown 6, 8 and 11 d under irradiance of 65, 130, 195 and 260 W m^{-2}. The extracts were analyzed under non-denaturing conditions on 10 % PAGE gels and stained either for peroxidase activity (*A*) or immunodetected using specific anti-srPRX serum (*B*). Each lane was loaded with 25 μg of total proteins. Lane TNV was loaded with 10 μg of the ICF proteins extracted from TNV-infected cucumber cotyledons.

Discussion

Continuous irradiation of photosynthetically active seedlings (a period of 4-11 d after sowing) with white light (130 W m^{-2}) dramatically enhanced the total soluble peroxidase activity. The same effect was observed when the corresponding extracts were analysed by anodic PAGE and Western blotting using highly specific polyclonal antiserum. In comparison with seedlings grown at 12-h photoperiod all

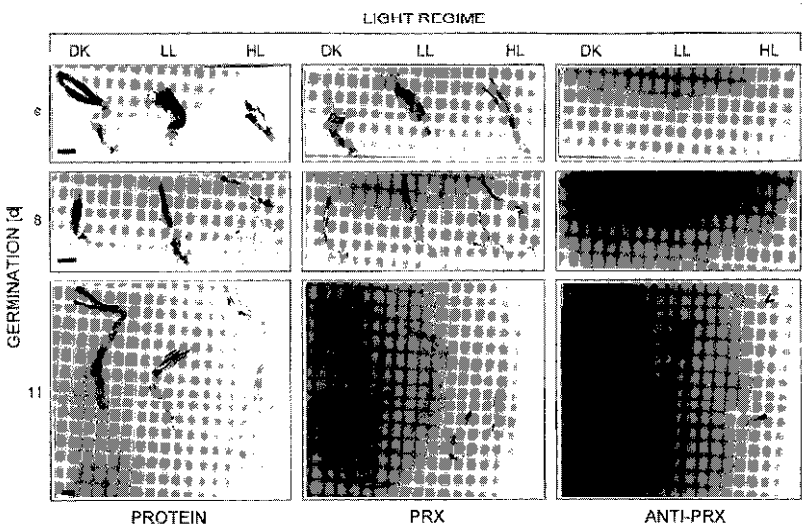

Fig. 5. Tissue prints from 6-, 8- and 11-d-old cucumber seedlings germinated under constant darkness (DK), irradiance 130 W m^{-2} (LL), irradiance 260 W m^{-2} (HL). The prints were stained either for total protein, total peroxidase or immunodecorated using anti-srPRX serum. *Arrowheads* (full and open) and *star* indicate the concentrated immunospecific signals. *Bars* 500 mm.

three srPRX isoenzymes were accumulated. Light-induced expression of srPRX was not unexpected because there is a set of experiments demonstrating that a minimal irradiance and photoperiod seem a prerequsite for optimal PR-protein accumulation induced by various chemicals (Asselin *et al.* 1985). Additionally, elicitors acting via ethylene, such as α aminobutyric acid, were shown to induce basic and acidic type PR-1 transcript accumulation in a light-dependent manner (Eyal *et al.*1992). Therefore we believe that light-induced expression of the srPRX is likely to be regulated in a similar way. In this context, two hypotheses may be considered to explain the expression of the srPRX in light-grown cucumber seedlings. One hypothesis assumes that light-induced expression of the srPRX could be mediated by phytochrome. There are several lines of evidence demonstrating the phytochrome-mediated effects on extracellular peroxidase activity (Casal *et al.* 1990, 1994). According to the second hypothesis, the accumulation of the srPRX isoenzymes could be induced by oxidative stress resulting from photoinhibition during exposure

of photosynthetically active seedlings to high irradiance. This statement is in a good agreement with the previous results of Khan and Malhotra (1982) and Grill *et al.* (1985) who reported that oxidative stress induced an increase in many forms of peroxidase. The generation of active oxygen species caused by exposure to high irradiance under decreased rate of CO_2 supply may also be important in the observed patterns of srPRX activity.

In contrast to light-induced accumulation of srPRX in photosynthetically active cucumber seedlings, the induction of the same set of isoenzymes, albeit in a lower extent, in dark was quite unexpected. Although Eyal *et al.* (1992) have shown that accumulation of a basic pathogenesis-related (PR-1) transcript was induced by dark, Asselin *et al.* (1985) have demonstrated that dark treatments of *Nicotiana* tissue almost completely inhibited PR-protein accumulation. On the basis of our previous results (Repka and Fischerová 1996) we suggest that likewise to light-induced accumulation of the srPRX, the expression of the same isoenzymes in dark-treated seedlings might be a consequence of oxidative stress resulting from high lipoxygenase (LOX) activity. Indeed, since the same isoenzymes are generated in the dark as well as the light indicate the possibility that the light-stimulated peroxidase isoenzyme activity is not related to photosynthesis *per se* but more probably to the oxidative stress perceived by the plant tissues. Apparently, without further experiments there is little that can be said about the actual physiological function of the srPRX in seedlings. But appears plausible that in both dark- and light-treated seedlings the induction pathway has the same origin.

Furthermore, we have studied the effect of various irradiances on the srPRX accumulation in cucumber seedlings. At irradiance of 195 and 260 W m^{-2} a marked difference in the expression of the particular srPRX isoenzymes was observed. Since this different expression is probably related to tissue-specific localization of particular isoenzymes, tissue printing experiments followed by immunolocalization of specific antigens were performed. These experiments revealed that the srPRX or its three molecular forms are differently spatially and temporally distributed in seedling grown under various irradiances. The similar patterns of distribution was observed in seedlings grown at 12-h ptotoperiod (Repka *et al.* 1997). As we have shown previously, the immunopositive signals found either in the cotyledons or in the transition zone was contributed strictly to srPRX1 isozyme (Repka and Fischerová 1996, Repka *et al.* 1997). Based on these works we have proposed that localization of srPRX 1 isoenzyme in the lower epidermis of growing cotyledons might be connected with deposition of the structural biopolymers like lignin, extensin, suberin or cutin (Fry 1986, Robb *et al.* 1991). In this context, immunolocalization of the srPRX in the epidermal tissue of cotyledons might be at least partially related to effect of ethylene. First, Repka and Vanek (1993) have shown that srPRX is strongly induced by ethrel (an ethylene releasing compound). Second, Cassab *et al.* (1988) have demonstrated that ethylene treatment of pea seedlings for 72 and 96 h changed the distribution pattern of both peroxidase and extensin. From this point of view it is important that ethylene induced accumulation of pea peroxidase was detected just in epidermal and cortical cells. There is also another important conclusion from the experiment dealing immunolocalization of the srPRX in seedlings germinated under

different light regimes. Since there is a substantial difference between the patterns of distribution of srPRX in etiolated and light-grown seedlings, the expression seems to be light controlled or regulated. Light effects in plants are often mediated by photoreceptors, particularly the red/far red-absorbing phytochrome (Tobin and Silverthorne 1985). Phytochrome control of extracellular peroxidase activity in mustard internodes was well documented by Casal *et al.* (1990). On the other hand, since etiolated seedlings did not performed active photoperception, we conclude that in this case the phytochrome is not a major component of the dark-regulated accumulation of the srPRX in cucumber.

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