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The substrate preference and histochemical localization argue against the direct role of cucumber stress-related anionic peroxidase in lignification

V. REPKA, D. ŠTETKOVÁ and I. FISCHEROVÁ

Laboratory of Molecular Biology and Virology, Research Institute of Viticulture and Enology, (C.R.I.V.E.), Matúškova 25, SK-83311 Bratislava, Slovakia

Abstract

The substrate preference and the localization of cucumber (*Cucumis sativus* L.) stress-related anionic peroxidase (srPRX) were investigated in order to assess whether this activity correlates with the lignification. The results showed that none of the purified srPRX isoenzymes (PRX 1 - 3) could oxidize the lignin monomer analog syringaldazine. The srPRX immunospecific signal was found to be highly abundant in both the extrafascicular and fascicular phloem regions in cucumber stem and leaf petiole. In *Nicotiana*, *Petunia* and *Dahlia*, the srPRX homologs were specifically deposited in both outer and inner phloem elements of stem and in both abaxial and adaxial phloem of leaf stems. The srPRX mRNA expression analysis showed similar pattern as for immunolocalization. The subcellular localization of immunospecific srPRX demonstrated that at least part of the peroxidase could be ionically-bound to phloem cell wall.

Additional key words: *Cucumis*, *Dahlia*, electrophoresis, immunoblotting, *Nicotiana*, tissue print Western, tissue print Northern.

Introduction

Plant peroxidases (EC 1.11.1.7) are multifunctional enzymes existing in a numerous molecular forms. There is an extensive literature indicating a role of peroxidase in a diverse array of developmentally regulated processes, probably due to involvement of these enzymes in auxin metabolism (IAA-oxidation, for a review see Cella and Carbonera 1997). Moreover, previous observations had already evidenced that peroxidase isoenzymes are organ- and/or tissue-specific (Hendriks and Van Loon 1990, Repka and Jung 1995, Repka and Fischerová 1996); usually their expression is developmentally controlled (Repka *et al.* 1997).

It has now become evident that besides growth and morphogenesis, peroxidase plays a crucial role in a plethora of physiological events of potential importance in plant-pathogen interactions. The best-documented examples include phenol oxidation (Lagrimini 1991), cross-linking of cell-wall components (Bradley *et al.* 1992), wound-healing (Sherf *et al.* 1993), systemic acquired resistance response (Irving and Kuc 1990), and particularly lignification (Walter 1992). The latter is a matter of special interest since as peroxidases are involved in lignin biosynthesis of wild type plants, increased peroxidase activity can cause a massive lignification of infected tissues that result in necrotic lesions during hypersensitive response of plants to pathogen attack. Correspondingly, a general consensus holds that a major function of anionic, extracellular peroxidase concerns lignin synthesis (Gaspar *et al.* 1982). Thus, rapid lignin deposition may provide a physical and/or chemical barrier to the invading pathogens.

Cucumber stress-related peroxidase (srPRX) is a soluble extracellular enzyme, most prominently accumulated in the apoplast of hypersensitively reacting cotyledons (Repka and Slováková 1994). Although various aspects of srPRX expression have been very thoroughly studied in relation to the stress response (Repka *et al.* 1996, Repka and Fischerová 1998,

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Abbreviations: ABTS - 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), BSA - bovine serum albumine, DAB - 3,3′-diaminobenzidine, ECL - enhanced chemiluminiscence, EDTA - ethylenediaminetetraacetic acid, GUA - guaiacol, HRGP - hydroxyproline-rich glycoprotein, ICF - intercellular fluid, NC - nitrocellulose, NY - nylon, PAGE - polyacrylamide gel electrophoresis, PRX - peroxidase, PVP - polyvinylpyrrolidone, SDS - sodium dodecylsulphate, srPRX - stress-related peroxidase, SYR - syringaldazine, TBS - Tris-buffered saline, TNV - tobacco necrosis virus.

Fax: (+421) 754 775436, e-mail: repka@crive.axonpro.sk

Repka and Fischerová 2000), remarkably little work has been done to characterize a potential role of this enzyme in the final polymerization step of lignin synthesis. To address this issue, we have focused here on the substrate preference and histochemical localization of srPRX in plant tissues.

Materials and methods

Plants: Cucumber (*Cucumis sativus* L. cv. Laura), tobacco (*Nicotiana tabacum* L. cv. White Burley), petunia (*Petunia hybrida* L. inbred line R27), and dahlia (*Dahlia variabilis* L.) plants were grown from seeds in the campus glasshouse (temperature 20 - 32 °C). Two-month-old plants and 9- to 16-d-old seedlings (cucumber) were used in all experiments. For tissue printing the internodes were arbitrarily numbered according to the order from young (top) to older (bottom). Potted plants were watered and fertilized as described elsewhere (Repka *et al.* 1996).

Protein extraction: Stems from 2-month-old cucumbers were harvested and ground with a pestle and mortar in appropriate volume of ice cold *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). The extract was centrifuged at 15 000 *g* for 20 min at 4 °C. The clear supernatant was further precipitated with acetone at -20 °C overnight. Precipitate was solubilized with electrophoresis sample buffer (Laemmli 1970) and stored at -20 °C for future use. Proteins ionically bound to cell walls were extracted basically according to Ros-Barceló *et al.* (1987), precipitated as described above and stored at -20 °C.

Exudate sampling: Phloem exudate samples were collected with micropipette from the basal stem ends in order to exclude a contribution from the xylem sap. Alternatively, exudate was pooled directly from cucumber seedlings stem cuttings immersed in *TRISEPAC* buffer (pH 8.0) to prevent air oxidation. For the collection of xylem exudates, plants were cut with a razor blade at the stem base. Residual phloem sap was blotted from the cut stump for 1 min, after which time the root pressure exudate continued to accumulate on the cut surface. Approximately, 0.05 cm^3 of root pressure exudate was collected from each of twelve cucumber plants and then added to Eppendorf tube containing an equal amount of reducing sample buffer (Laemmli 1970). Both types of exudate were stored at -20 °C until use.

Sample analysis: The protein concentration in the tissue extracts and exudates was determined by the method of Bradford (1976), using BSA as a standard.

Peroxidase purification: The stress-related anionic PRX isoforms (srPRX 1, 2 and 3) were purified to an electrophoretical homogeneity from ICF of hyper-sensitively reacting cucumber cotyledons. Approximately 200 cm^3 of ICF was pooled from TNV-infected cotyledons by vacuum infiltration procedure and peroxidases were purified following a previously published protocol (Repka and Slováková 1994).

Peroxidase assays: Peroxidase activity was measured on *Shimadzu UV-1601* spectrophotometer (*Shimadzu Europe Ltd.*, Manchester, UK) at 20 °C following the H_2O_2-dependent oxidation of GUA at 470 nm. The reaction mixture contained 50 mM sodium acetate buffer (pH 5.2), 0.3 % GUA, and 0.3 % H_2O_2. For ABTS assay, peroxidase activity was measured spectrophotometrically at 20 °C by determining the H_2O_2-dependent oxidation of ABTS at 418 nm. The reaction mixture contained 20 mM sodium citrate buffer (pH 5.5), 0.04 % ABTS (*Sigma GmbH.*, Deisenhofen, Germany), and 0.006 % H_2O_2.

For the SYR assay, peroxidase activity was measured spectrophotometrically at 20 °C by determining the initial rate of the H_2O_2-dependent oxidation of SYR at 530 nm. The reaction mixture contained 5 mM Tris-HCl buffer (pH 7.5), 20 μM SYR (*Sigma GmbH.*, Deisenhofen, Germany), and 0.03 % H_2O_2. The method was adapted from that of Goldberg *et al.*(1983).

For both qualitative and quantitative screening of plant extracts, the slot-blot technique was employed. In all cases, equal aliquots of plant extracts were applied with membranes installed in the slot-blot apparatus *PR 648* (*Hoefer Scientific, Inc.*, San Francisco, USA). Following a short incubation and removal of protein solution by suction, the membrane was washed in TBS buffer and immersed in a PRX chemiluminscence substrate (*SuperSignal West Dura, Pierce Inc.*, Rockford, USA) for 5 min. Then the membrane was sealed in a plastic protector and exposed to *ECL-Hyperfilm* (*Amersham plc.*, Buckinghamshire, UK).

Analytical PAGE: Reduced proteins were fractionated by SDS-PAGE using a 4 % stacking gel and 12.5 % separating gel as described by Laemmli (1970). Each lane of the gel was loaded with equal amount of protein and bromophenol blue as marker. Gels were subsequently fixed overnight and silver stained according to Blum *et al.* (1987).

Western blotting: After the separation, proteins were electroblotted onto NC-membrane (*PROTRAN BA-85,*

0.45 μm, *Schleicher and Schuell,* Dassell, Germany) at 4 °C for 20 h at 50 mA using 40 mM phosphate buffer (pH 6.5) as a transfer medium. After the blocking of membrane with 5 % non-fat dry milk (*Blotto)*, blots were incubated for 1 h with *Cucumis sativus* srPRX (1:1000) polyclonal antibody. Horse radish peroxidase-conjugated swine anti-rabbit IgG (SWaR, *Sevac,* Prague, Czech Republic) was diluted 1:50 000 for the secondary antibody reaction. Immunospecific signal was visualized with enhanced chemiluminiscence using *SuperSignal™ West Dura* kit (*Pierce Inc.*, Rockford, USA) on *Hyperfilm-ECL* (*Amersham*, Buckinghamshire, UK).

Tissue print Western: Tissue print immunoblots were performed basically according to a modified protocol of Cassab and Varner (1987). Fresh hand-sections (1 mm) were carefully transferred on the NC-membrane and pressed gently and evenly for 20 s. At least four replicate prints were made from the same age materials. Following the printing, the membrane was baked at least for 8 h at 80 °C and immunoprocessed according our standard Western blotting protocol (Repka and Slováková 1994). For localization of srPRX, rabbit polyclonal antibody against cucumber srPRX was used (dilution 1:1 000). Horse radish-conjugated swine anti-rabbit antibody (SWaR, *Sevac*, Prague, Czech Republic, dilution 1:50 000) was used as the secondary antibody. Immunospecific signal was developed using the *SuperSignal West Dura* chemiluminiscence kit (*Pierce Inc.*, Rockford, USA). The membrane was exposed to *Hyperfilm-ECL* (*Amersham*, Buckinghamshire, UK) at room temperature for varying duration of time and then the films were processed using standard X-ray developer and fixer.

Tissue print Northern: Nylon membranes (*NYTRAN N-13*, *Schleicher and Schuell*, Dassell, Germany) were used for tissue print RNA blots without pretreatment according to the protocols described by McClure and Guilfoyle (1989) and Varner *et al.* (1988). Tissue prints were performed the same way as described for tissue print immunoblots. After printing, the membrane was illuminated with short-wave (302 nm) UV light using *UV Crosslinker* (*UVP, Inc.*, Upland, USA) for 20 min to fixing the nucleic acids on the membrane. Then, the membrane was washed in 0.2 × SSPE (6 × SSPE = 900 mM NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.7) and 1 % SDS for 4 h at 47 °C. The membrane was prehybridized for 17 h at 42 °C in nuclease-free *BLOTTO-MF* solution (120 mM Tris-HCl, pH 7.4, 8 mM EDTA, 600 mM NaCl, 1 % non-fat dried milk powder, 50 % deionized formamide, 1 % SDS) according to Monstein *et al.* (1992). A synthetic oligonucleotide (21-mer, *MWG Biotech AG*, Ebersberg, Germany) modified at the 5′end with biotin was used as the RNA complementary probe. The design of the probe was derived from the nucleotide sequence of the coding strand for the peptide sequence HFHDCFV (5′- CATTTTCACGATTGTTTCGT - 3′, Henrisatt *et al.* 1990). Hybridization of the probe (100 ng cm^{-3}) to the membrane was carried out in *BLOTTO-MF* solution at 46 °C for 24 h. Posthybridization stringency washes consisted of three 15 - min washes in 6 × SSPE, 0.1 % SDS at 25 °C, one 15 - min wash in 6 × SSPE, 0.1 % SDS at 46 °C, and one 15-min wash in 6 × SSPE at 46 °C.

Membranes hybridized with biotinylated probe were incubated at 25 °C for 1 h in 5 % *BLOTTO-TEN* buffer (Repka and Slováková 1994). Membranes were then incubated in a solution of horse radish peroxidase-conjugated avidin D (2.5 μg cm^{-3}, *Vector Laboratories, Inc.*, Burlingame, USA) in TBS buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 0.05 % Tween-20) for 1 h. Finally, the membranes were washed with three 10-min changes of TBS buffer. Colour development was effected by incubating the membranes in TBS buffer containing 0.03 % DAB (*Amresco*, Solon, USA), 0.03 % H_2O_2 and intensifier (0.03 % $CoCl_2$/$NiCl_2$). Staining reaction was terminated by intensive washing the filters with distilled water.

Total nucleic acids on the membrane were visualized for comparison with specific signal either with silver staining protocol according to Su (1987) or using the *GenoGold* kit (*Vector Laboratories,Inc.*, Burlingame, USA).

Histochemical assays: Histochemical tests were performed either directly on the fresh hand-made sections or after the tissue printing on NC-membrane. Staining for anatomy comparison, lignin and H_2O_2 was done on fresh tissues immediately after the sectioning. The section was stained with 0.025 % toluidine blue (*Sigma*, Deisenhofen, Germany) to show the anatomy. Lignin was stained histochemically with 2 % phloroglucinol (*Sigma*, Deisenhofen, Germany) in 7.5 - 30 % HCl (Jensen 1962). The presence and localization of H_2O_2 in living tissue were assayed according to recently developed enhanced protocol using 1 % $KMnO_4$ (Repka 1999).

Tissue prints on NC-membrane were used to show either a total protein (1 % Amido Black) or a total soluble peroxidase (0.03 % DAB) according to Repka and Fischerová (1998). The cell wall-associated peroxidase was assayed on a NC-membrane pretreated with 0.2 M $CaCl_2$ for 30 min and air dried.

Results

Enzymatic properties of cucumber peroxidases from fractionated plant extracts: In the initial experiment the rate and distribution pattern of peroxidase activity in the fractionated plant extracts were measured. We assayed peroxidase activity in the presence of both artificial (GUA and ABTS) and natural (SYR) hydrogen donors. The results showed that only the GUA gave the complex pattern of peroxidase distribution (Fig. 1*A*). ABTS-oxidizing peroxidase was localized more strictly either in the low-salt extract of cucumber stem tissue or in the phloem sap. The SYR-oxidizing activity was restricted to low-salt extract and it was not present in the others, even though the high endogenous PRX activity was detected in either of plant extracts (Fig.1*B*). Thus, SYR-peroxidase seems to be confined to soluble and/or covalently-bound fraction.

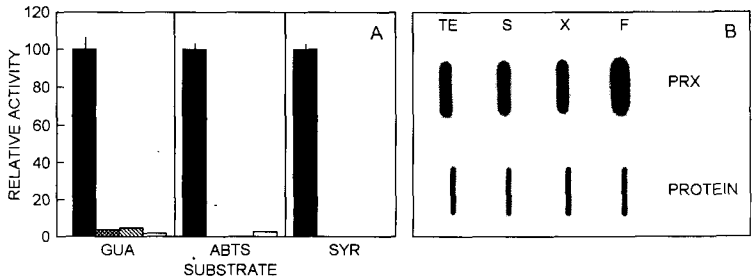

Fig. 1. Analysis of the substrate specificity of soluble, ionically-bound, xylem-localized, and phloem-specific peroxidase (*full, dotted, hatched,* and *open columns*, respectively) extracted from vegetative cucumber plants (*A*). The activity of the most active extract for each substrate was set to 100. The measurements were repeated four times and *bars* indicate SE. An unspecific total soluble PRX (10 μg per slot) present in either extract assayed by chemiluminiscence was included for comparison (*B*). The total protein slot-blot (10 μg per slot) being used as a loading control. (TE - total low-salt extract, S - high-salt extract, X - xylem exudate, F - phloem exudate).

Substrate preference of purified molecular forms of the srPRX: To test a putative lignin-specific activity of the major stress-related anionic peroxidase of cucumber, its three molecular forms (isoenzymes) were first purified to an electrophoretical homogeneity from intercellular fluid. Substrate preference assays showed that all three isoenzymes could readily oxidize both GUA and ABTS, albeit to a slightly different extent. When the naturally-occuring substrate (SYR) was used as the hydrogen donor none from the purified isoforms of srPRX had the capability to oxidize it (Fig. 2).

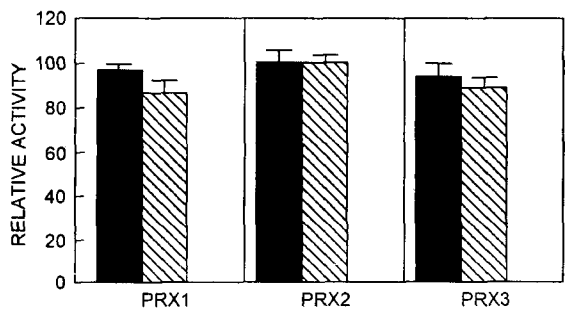

Fig. 2. Analysis of the substrate preference of purified srPRX isoenzymes. The rate of oxidation for the three substrates GUA (*full columns*), ABTS (*hatched columns*) and SYR (*open columns*) was measured for the three purified isoenzymes. The activity of the most active isoenzyme for each substrate was set to 100. Values represent the means of four separate measurements, *bars* indicate SE.

Immunohistochemical localization of srPRX in developing cucumber tissues: We have used tissue print immunoblots to localize srPRX in developing cucumber tissues. For nomenclature, consistent with the resolution of the localization results, the phloem anatomy of stems is shown in Fig. 3.

In the young cucumber stem, srPRX is heavily accumulated in the internal and external bundle phloem (Fig. 4*F*). Furthermore, immunospecific signal appeared in the cortical extrafascicular phloem and more precisely in the entocyclic phloem strand. In the oldest internode, close to the zone of transition between the shoot and roots, an immunospecific signal remains to be localized in the fascicular phloem located both internally and externally to the xylem (Fig. 4*L*). The pattern of srPRX deposition in the leaf petiole resembles that typical for old stem immunoprint (Fig. 4*S*). Interestingly, the distribution of immune-specific signal did not correlate with the pattern of total soluble peroxidase localization (Fig. 4*E,K,R*). In this case, DAB-specific peroxidase appeared mainly in the sclerenchyma ring (Fig. 4*E*) and in the epidermis (Fig. 4*E,R*). Moreover, in the old stem print (Fig. 4*K*), the extracellularly localized peroxidase was heavily stained. The results also shown that immunospecific signal did not co-localize neither with total soluble peroxidase nor both lignin (Fig. 4*B,H,N*) and H_2O_2 (Fig. 4*C,I,O*) deposition.

Immunolocalization of the srPRX-related protein in tobacco, petunia and dahlia: We examined the tissue specific localization of the srPRX-related protein in two *Solanaceae* plants, *i.e.* tobacco and petunia and one *Asteraceae* plant, dahlia (*Dahlia variabilis* L.). The immunolocalization (Fig. 5) confirmed the phloem-specific deposition of srPRX, albeit a quantitative difference in the signal can be seen.

In tobacco, srPRX immune signal was accumulated mainly in the outer and inner phloem regions of the stem as well as in the abaxial and adaxial phloem regions of leaf midvein. In petunia, srPRX immune signal was present mostly in the outer phloem region of the stem and in the abaxial phloem region of leaf midvein. In dahlia stem and petiole, srPRX immune signal was most abundant in the primary phloem region and bundle-associated extrafascicular phloem, respectively. These figures also showed an asymmetric distribution of srPRX immune signals in the stem cross-sections with the least srPRX on the side having the leaf.

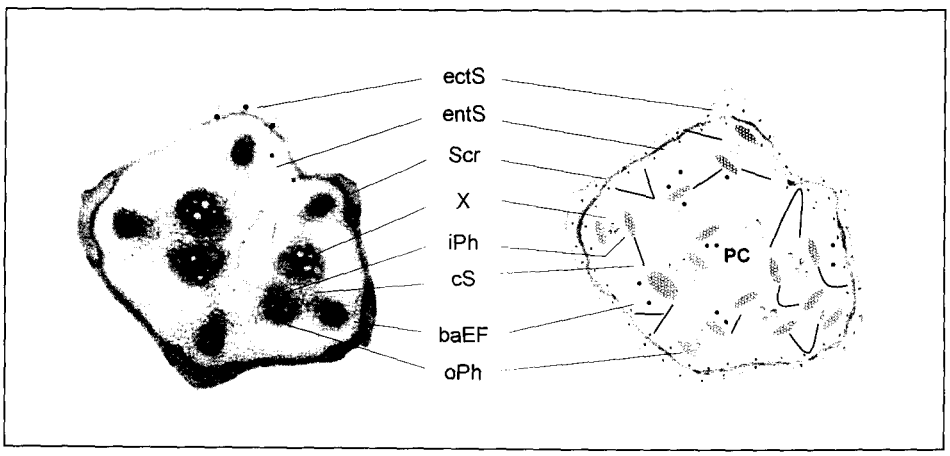

Fig. 3. The vascular anatomy of a transverse section of *Cucumis sativus* L stem. Bicollateral vascular bundles are composed of internal (*iPh*) and external (*oPh*) fascicular phloem flanking the xylem (*X*) on two sides. The extrafascicular phloem consits of entocyclic (*entS*) and ectocyclic (*ectS*) phloem strands separated by a sclerenchyma (*Scr*) ring in the cortex and commisural sieve elements (*cS*) forming lateral connections between the longitudinal phloem strands. Bundle-associated extrafascicular phloem (*baEF*) is located in arcs bordering the internal and external bundle phloem. A pith cavity (*PC*) is present in *Cucumis* spp. stem.

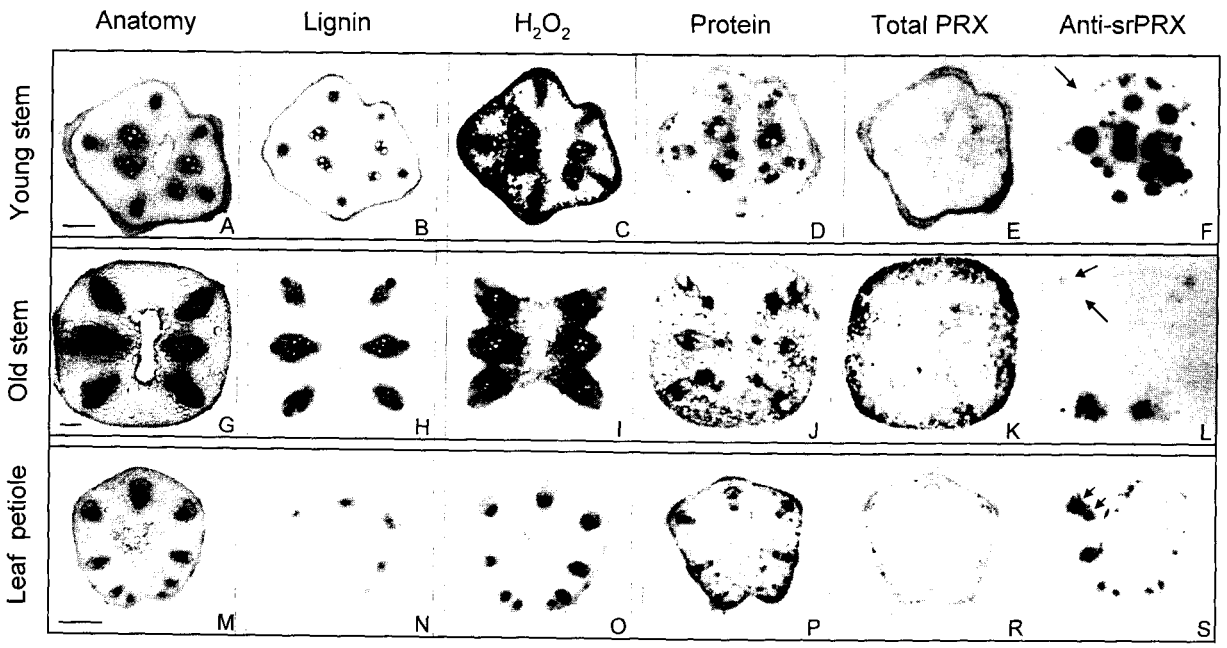

Fig. 4. Tissue-print immunolocalization of srPRX in cucumber stems and leaf. *Cucumis sativus* srPRX antibody reacted with tissue-prints from (*F*) young stem, (*L*) old stem, and from (*S*) leaf petiole. *Arrows* in (F), (L), and (S) indicate extrafascicular and bundle-associated phloem, respectively. For comparison, the same age tissue sections were stained for anatomy, lignin content, H_2O_2, total protein and total PRX (*bars* - 300 μm in all sections).

Gene expression of srPRX in plants: To know whether the srPRX mRNA expression pattern is similar to that for the srPRX protein we have performed tissue print hybridization in tobacco and petunia. In tobacco stem, srPRX gene was most abundantly expressed in the outer phloem region (Fig. 6*B,D*) and also in the inner phloem region but only in young stem (Fig. 6*B*). In petunia, srPRX mRNA was also present mostly in the outer phloem region of the stem (Fig. 6*F*). Likewise to tissue print immunoblots, these figures confirmed an asymmetric distribution of these mRNAs in the stem cross-sections with the least mRNAs on the side having the leaf.

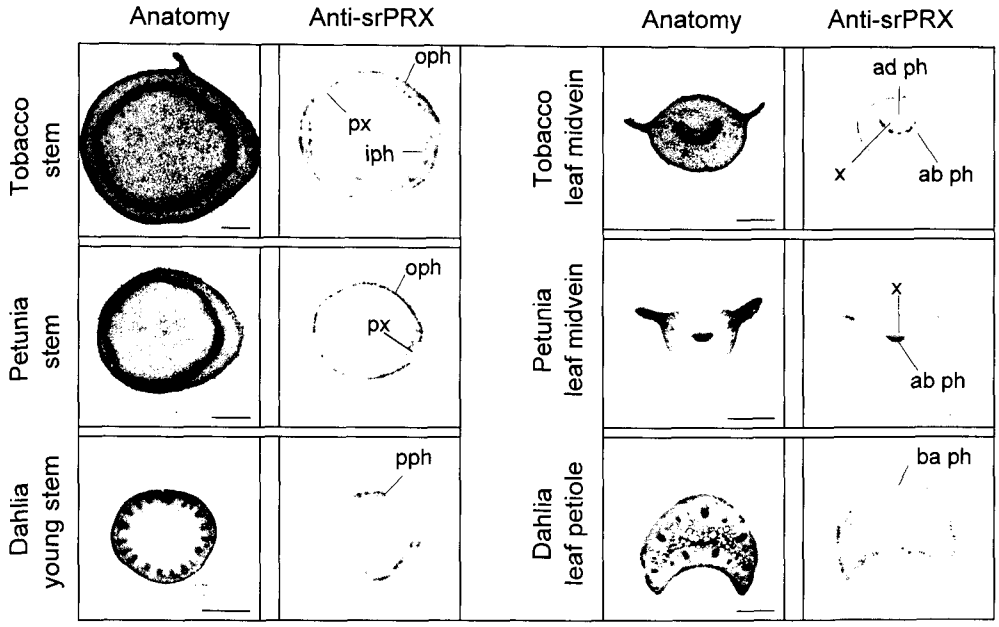

Fig. 5. Tissue-print immunolocalization of srPRX homologous protein in tobacco, petunia and *Dahlia* stems and leaves. The cucumber srPRX antibody was used for immunodetection. The figures are oriented so that in every case the side of the stem cross-section which subtend the leaf is up (*oph* - outer phloem, *iph* - inner phloem, *px* - primary xylem, *pph* - primary phloem, *adph* - adaxial phloem, *abph* - abaxial phloem, *x* - xylem, *baph* - bundle-associated phloem). *Scale bars* equal 300 μm in all sections.

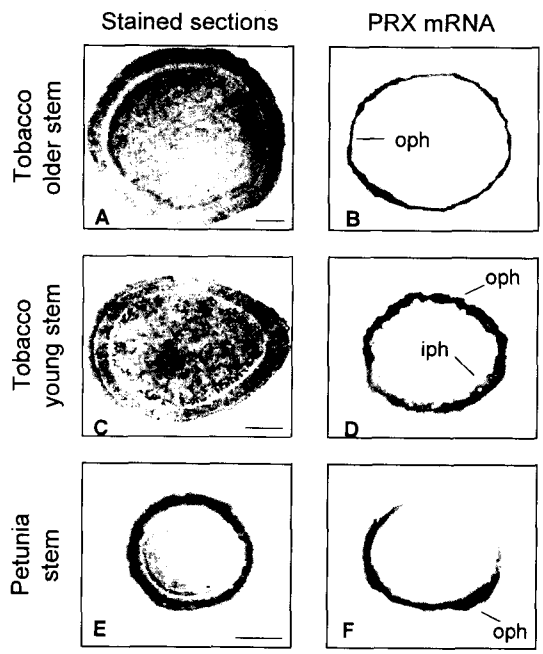

Fig. 6. Tissue print hybridization localization of srPRX mRNA in tobacco and petunia stems. The hybridized sections were stained with *GenoGold* kit to show total nucleic acids. The figures are oriented so that in every case the side of the stem cross-section which subtend the leaf is on the left (*oph* - outer phloem, *iph* - inner phloem). *Scale bars* equal 300 μm in all sections.

Subcellular localization of srPRX in cucumber stem: The low-salt extract, as well as the phloem and xylem exudates immediately were collected for SDS-PAGE and immunoblot analysis (Fig. 7*A,B*). Polyclonal antibody to the purified, extracellular anionic srPRX of cucumber were used to distinguish definitively among srPRX homologs in fractionated plant extracts. Indeed, the specific srPRX protein was detected readily in low-salt total extract and in the lanes containing the each of the purified isoforms of srPRX, whereas neither protein was immunodetected among total phloem and xylem exudate proteins (Fig. 7*B*).

Thereafter, total plant extract was further fractionated to reveal the immunoblot patterns of soluble and ionically-bound cell wall homolog of srPRX proteins. The results (Fig. 7*C,D*) illustrate the presence of srPRX specific immune signal in both extracts, even when with a marked quantitative difference. The immunospecific signal was at least two times more intense in the low-salt soluble extract than that in the the high-salt one. These results clearly indicate that, albeit a small fraction of srPRX protein may be, perhaps transiently, bound to the cell wall compartment.

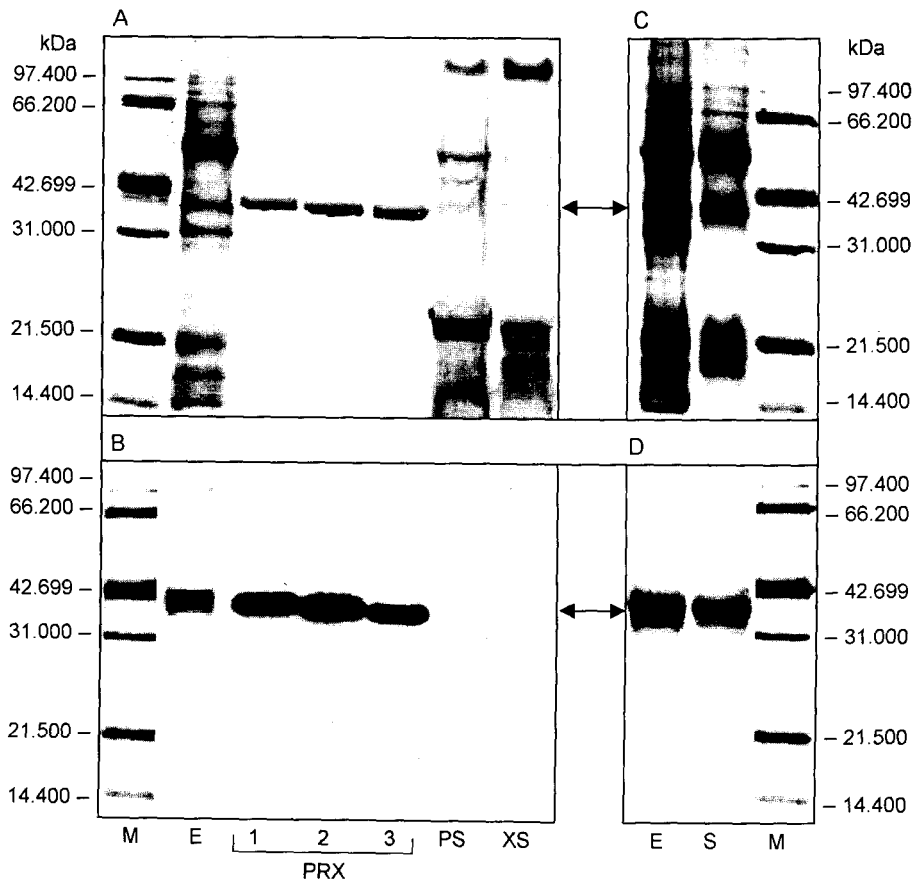

Fig. 7. SDS-PAGE and immunoblot analysis of *Cucumis* fractionated plant extracts and exudates. Protein patterns of soluble (*lane E*) and exudate (*PS* - phloem sap, *XS* - xylem sap) fractions were detected with silver staining (*A*). One microgramme amounts of each of purified srPRX (PRX 1 - 3) were separated along with the extracts. Immunoblot of duplicate gel reacted with polyclonal antibody raised against the srPRX (*B*). Low-salt (*lane E*) and high-salt (*lane S*) extracted proteins were compared to identify soluble and ionically-bound forms of srPRX. Silver stained (*C*) and corresponding immunoblot (*D*). Besides purified srPRX isoforms, each lane of the gel was loaded with 10 and 20 μg protein for silver staining and Western blot, respectively. *Arrows* indicate the position of the srPRX protein on the gel or blot, and *M* indicates prestained molecular mass markers.

Discussion

We have previously shown (Repka and Slováková 1994) that all of the srPRX isoenzymes are the fast-migrating, low-salt-extratable and highly anionic (*pI* around 3.4) isoenzymes. Since it has frequently been suggested that anionic peroxidases with similar characteristics are involved in the formation of lignin (Mäder *et al.* 1975, 1977), we have begun a search whether the peroxidases isolated here are potentially involved in lignification.

To identify putative lignin-specific peroxidases, a substrate preference analysis of fractionated plant extracts was performed as a unique starting point. By comparing the substrate-specific soluble PRX activity in tissue extracts and in phloem and xylem exudates, it was found that only total, low-salt extract of cucumber stem contained PRX with SYR-oxidizing activity. SYR was used as the only natural substrate in our tests because oxidation of SYR has been correlated with lignification in a wide range of plant species (Harkin and Obst 1973, Pang *et al.* 1989, Christensen *et al.* 1998). Moreover, kinetic measurements taken with SYR and/or syringaldehyde closely resembles it as an abundant lignin precursor also in cucumber (data not shown). Interestingly, Goldberg *et al.* (1983) observed identical K_M values for the SYR-oxidizing activities in both the cytoplasmic (soluble) and the cell-wall (bound) fractions. In cucumber, SYR-oxidase activity was observed only in low-salt buffer extract. Such a discrepancy could be due to the fact that corresponding PRX was probably not ionically- but covalently-bound to the cell wall. Whether or not it is the case, subsequent reextraction after digestion with cell wall-degrading enzymes should be performed.

Surprisingly, none of the purified isoenzymes was capable of SYR-oxidation although all isoenzymes readily oxidized both artificial substrates (GUA and ABTS). These data suggest that these isoenzymes are not functionally and/or genetically homologous to the peroxidase isoenzymes believed to be involved in lignification, *e.g.* in tobacco. In this context, there are two indirect and independent findings both originated from a transgenic plants, which support our statement. First, Lagrimini (1991) demonstrated that tobacco plants transformed with a chimeric tobacco anionic *prx* gene synthesize high levels of PRX in all tissues throughout the plant. Moreover, the percentage of lignin and lignin-related polymers in cell walls was nearly twofold greater in pith parenchyma tissue isolated from PRX-overproducer plant compared to control (untransformed) plant. In contrast to this, Ray *et al.* (1998) recently reported that increased PRX activity in potato plants transformed with a cucumber anionic peroxidase had no effect on the levels of soluble phenolics or the amount of lignin. Thus, if their anionic PRX which shares a common characteristics with our one could be a putative lignin-peroxidase, the amount of lignin-like material should be correlated with the rate of srPRX expression.

Immunohistochemical localization with the antibody against the cucumber srPRX demonstrated that the srPRX was primarily localized in the phloem elements (extrafascicular phloem and bundle-associated phloem) in cucumber stem and leaf petiole. It is important to note that tissue-printing results showed also a strong cross-reaction with antibody against cucumber srPRX in three other plant species belonging to two families (*Solanaceae* and *Asteraceae*). The localization of both the srPRX immune signal and the srPRX mRNA in phloem of tobacco, petunia and dahlia stems and petioles indicated the possibility that there is a more general pattern of srPRX. Additionally, there are two important kinds of view dealing the observed common patterns of srPRX expression. First, phloem-specific localization of both the srPRX protein and the srPRX mRNA showed an asymmetric distribution in the stem cross-sections with the least of the signal on the side having the leaf. This was not due to tissue print artifact, because all four tissue print repeats from each sample showed the identical pattern. Second, the localization of srPRX protein and srPRX mRNA strongly correlated with the patterns described for HRGP (extensin) in tobacco, tomato and petunia (Ye *et al.* 1991). The expression of the HRGP mRNAs in *Solanaceae* plants was generally more abundant in outer than in inner phloem and likewise for srPRX showed an asymmetric distribution. Taken together, these data indicate that both the srPRX and the HRGP may be needed in the formation of the wall architecture during differentiation of the sieve-tube member. Moreover, based on its enzymatic characteristic and phloem-specific localization, srPRX function can be causally interlocked with this compartment.

Two hypotheses are offered that might explain the possible function(s) of srPRX. Due to strong correlation between the srPRX and extensin localization, it is obvious that srPRX may be effective in PRX-mediated interpolypeptide cross-linking of extensin via isodityrosine bridges (Fry 1986). Indeed, Zheng and Van Huystee (1991) demonstrated that anionic PRX from peanut cell culture medium possessed the capacity to oxidize tyrosine to dityrosine, isodityrosine and polytyrosine. Thus, isodityrosine bridges may be responsible for the construction of a structural network contributing to the cell wall architecture, especially under the stress situations. It is well known that several external stresses, including mechanical wounding, elicitor treatment, and infection, can induce or increase the expression of both the anionic PRX and the extensin (Showalter and Varner 1989, Repka and Slováková 1994). The second suggestion is that the srPRX could assist either intra- or intermolecular cross-linking of phloem-specific macromolecules like P-proteins. The biochemistry of P-proteins is best characterized from members of the *Cucurbitaceae*, where they are produced in abundance in exudate formed on wounding (Sabnis and Hart 1976, 1979). An interesting feature is that P-proteins readily oxidize at wound surface (Read and Northcote 1983) and thus, it seems plausible that change in the redox state could play a regulatory role in aggregating P-protein filaments. Taking into account the fact that peroxidase itself can work as an oxidase (Morel *et al.*

1991), one could imagine the situation that srPRX may be instrumental in the mechanism regulating gel-sol transitions between P-protein subunits and filaments based on the change of the redox state (Alosi *et al.* 1988). There are at least two functional aspects resulted from a rapid oxidative polymerization of P-proteins in the phloem compartment. Aggregated P-proteins can serve as a plug blocking either leakage of assimilates at wound surfaces (Read and Northcote 1983) or, at least partially, a long-distance (systematic) spread of certain viruses in infected plants (Taliansky and Garcia-Arenal 1995). Although to confirm such a suggestion additional experiment are urgently needed, perhaps it is not an accident that srPRX expression was shown to be highly induced either by wounding or by virus infection (Repka and Vanek 1993, Repka and Slováková 1994).

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