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Organ-specific expression of the stress-related anionic peroxidases in cucumber flowers

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Abstract

The study revealed a marked qualitative and quantitative differences in the pattern of expression of three stress-related cucumber (*Cucumis sativus* L., cv. Laura) isoperoxidases. Activity staining, as well as the protein gel blot (Western blotting) confirmed that the proteins studied are differentially expressed in both male and female sepals, in male but not in female pedicels and in pistil. By using antibodies specific to three stress-related peroxidases, one serologically related pistil-specific anionic peroxidase was detected. This specific band had never been observed in other flower organs. Differential appearance of the stress-related peroxidase isoenzymes in both male and female flowers demonstrates that these proteins are developmentally regulated, showing an organ-specific expression.

Key words: *Cucumis*, ovary, pedicel, petal, pistil, sepal, stamen, tobacco necrosis virus.

Introduction

Peroxidases represent a class of ubiquitous enzymes widely distributed throughout the plant kingdom and have been implicated in several biosynthetic and degradative metabolic functions (for review see Gaspar *et al.* 1982, Greppin *et al.* 1986, Van Huystee 1987, Dunford 1991, Welinder *et al.* 1993). Some specific peroxidases have been proved to be good physiological markers in such events as: floral initiation in shoot apices (Nakanishi and Fujii 1992), rooting performance in *in vitro* propagated

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Abbreviations: AGPs - arabinogalactan proteins; BSA - bovine serum albumine; EDTA - ethylene-diaminetetraacetic acid; HPRGs - hydroxyproline-rich glycoproteins (extensins); IAA - indole-3-acetic acid; NC - nitrocellulose; PAGE - polyacrylamide gel electrophoresis; PELPs - extensine-like proteins; PR - pathogenesis-related; PRPs - proline-rich proteins; PVP - polyvinylpyrrolidone; TNV - tobacco necrosis virus;

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shoots (Dalet and Cornu 1989, Gaspar *et al.*1992), sexual differentiation (Hirsch and Fortune 1984), as well as the somatic embryogenesis (Zhou *et al.* 1992).

We have previously shown that a set of three anionic peroxidases is induced and rapidly accumulated in cucumber cotyledons reacting hypersensitively to TNV inoculation (Repka *et al.* 1991). We have also shown that beside the virus infection the same set of peroxidase isoenzymes was induced by different biotic and abiotic stresses (Repka and Vanek 1993). Subsequently, these stress- or pathogenesis-related anionic peroxidases were purified to homogeneity and highly specific antisera against the purified proteins were raised (Repka and Slováková 1994).

In contrast to the coordinated expression of the PR-genes during pathogenesis, their expression appears to be differentially regulated in an organ- and tissue-specific manner during development (Felix and Meins 1986, Memelink *et al.* 1990). The developmental regulation of PR-genes implies that these also play a role in the physiology of healthy plants. Alternatively, the PR-proteins may have the similar functions during pathogenesis and normal plant development.

Our long term objective is to trace the various stress-related peroxidases. Studying their potential roles in normal development of cucumber plant may provide clues to understand their function in defense and *vice versa*. With this in mind, the most appealing approach for such study was to employ immunological assays (Western blotting).

Materials and methods

Plant material: Cucumber (*Cucumis sativus* L. cv. Laura) seeds were surface sterilized, germinated and grown *1*) 7 d in moist perlite at 28 °C in the dark or *2*) two months in sterilized soil in a glasshouse (temperature 20 - 32 °C). All plants were watered to saturation daily and fertilized bi-weekly with *OBM* fertilizer (NPK ratio 1.4:10:8).

Inoculation of plants: Cotyledons of *ca.* 7-d-old plants were abrased using carborundum and then infected with TNV virus (kindly supplied by Prof. Horváth) as described previously (Repka and Slováková 1994). Control plants were treated similarly with a homogenate of non-infected leaves or with virus-isolation buffer.

Organ preparation and protein isolation: Two-month-old flowering plants were used. Male and female flowers were harvested at anthesis and divided into major organs. Homogenates were obtained by grinding tissues in a prechilled mortar at 4 °C 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). After extraction (10 min at 4 °C), homogenates were centrifuged at 15 000 *g* for 20 min at 4 °C. Supernatant fractions were kept frozen at -20 °C.

Protein and peroxidase activity determination: Protein concentration in the homogenates was determined colorimetrically according to Bradford (1976) with BSA as the standard. Peroxidase activity was assayed at 20 °C using quaiacol as the

hydrogen donor according to Frič and Fuchs (1970). Enzymatic activity was expressed as change in absorbance at 470 nm [ΔA_{470} s^{-1} mg^{-1}(protein)].

Electrophoresis (PAGE) and staining of peroxidase isoenzymes: Peroxidase isoenzyme patterns were obtained by subjecting centrifuged extracts to native PAGE as described previously (Repka and Slováková 1994), followed by staining with 3,3-diaminobenzidine (*Sigma*) and H_2O_2 (0.05 % and 0.02 %, respectively).

Immunoblotting (Western blot): The electrophoretic transfer of proteins from non-denaturating PAGE gels to a NC-membrane (0.45 μm; *Schleicher and Schuell*, Dassel, Germany) was carried out at 4 °C for 20 h at 50 mA. Following the transfer, the membrane was baked at 80 °C for at least 8 h to inactivate endogenous peroxidases. After blocking the membrane with a solution containing either 2 % BSA or 5 % nonfat dry milk (*Blotto*), the Western blots were processed as described previously (Repka and Slováková 1994). For obtaining a complex picture about the expression of the stress-related peroxidases, a mixture of cucumber anti-PRX 1, 2 and 3 antibodies were used.

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

Results

The total soluble peroxidase activity in different parts of male and female flowers: Beside the TNV-infected cotyledon tissue, the highest peroxidase activity was

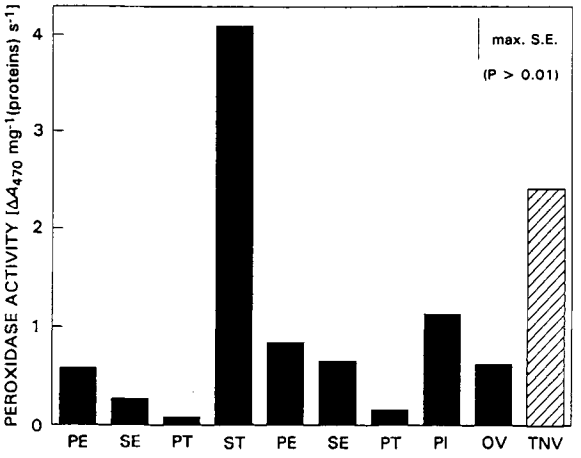

Fig. 1. The total soluble peroxidase activity in extracts from different organs of male and female flowers and from TNV infected cucumber cotyledons. Values are means of three separate measurements. PE - pedicel; SE - sepal; PT - petal; PI - pistil; OV - ovary; ST - stamen; TNV - tobacco necrosis virus.

determined in both reproductive flower organs, the stamen and pistil. A lower peroxidase activity observed in male, as well as in female flowers was typical for pedicel, sepal and petal extracts (Fig. 1).

Organ-specific expression of the stress-related peroxidases in cucumber flowers: Both male and female flower extracts (sepals) showed the three previously identified (Repka *et al.* 1993) acidic bands (formerly named as Prxl, 2 and 3) and exhibited the strongest signal (Fig. 2A). In male pedicels, the same peroxidase isoenzyme pattern was present but weaker than in sepals from both male and female flowers or from TNV-infected cotyledons. Unlike pedicels from male flowers, no acidic stress-related peroxidase isoenzymes could be detected in pedicels from female flowers. For the female flowers, a pistil-specific expression of the Prx3 isoenzyme was found. In addition to this major activity, one minor pistil-specific peroxidase band was

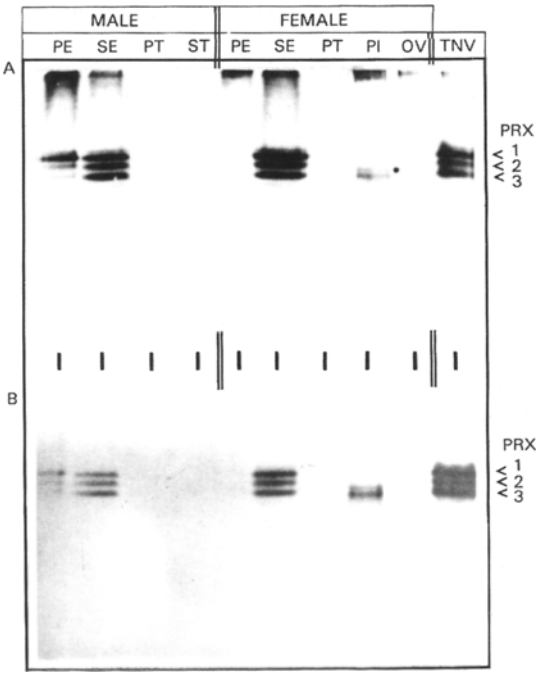

Fig. 2. Comparative 10 % polyacrylamide gel electrophoresis under native conditions. Proteins were extracted from organs as indicated, as well as from TNV-infected cucumber cotyledons. Each lane was loaded with 50 μg of proteins and gel was activity stained for isoperoxidase pattern (*A*) or transferred to NC-membrane and immunodecorated with specific antiserum raised against purified peroxidases (*B*). Black dot in pistil lane denotes a novel pistil-specific anodic peroxidase isoenzyme. PE - pedicel; SE - sepal; PT - petal; PI - pistil; OV - ovary; ST - stamen; TNV - tobacco necrosis virus.

detected. None acidic peroxidase activity was observed in male and female petals, stamen and ovary extracts.

Polyclonal antibodies to the purified, extracellular acidic proteins of TNV-infected cucumber cotyledons (Repka and Slováková 1994) were used to detect and quantify the amounts of the stress-related peroxidases using Western blots. Several immunologically cross-reacting protein species were observed clearly in male pedicel, male and female sepals, as well as in pistil (Fig. 2B). These results agree with those obtained for the distribution of the stress-related peroxidase isoenzymes on activity stained gel. Surprisingly, the minor pistil-specific peroxidase isoenzyme band serologically cross-reacted with the antibodies raised against the purified Prx1, 2 and 3 proteins.

Organ-specific distribution of Prx1,2 and 3 proteins: Distribution of the particular Prx isoenzymes present in the male and female sepals (Fig. 3) reflects the pattern of TNV-infected cotyledon tissues. Moreover, the relative abundance among the particular isoenzymes present in both male and female organs are about equal to that of TNV-infected cotyledons. The quite different situation was observed for pedicels. Unlike to the female organ, where the isoenzyme bands were not detected, in male pedicels a steep gradient of the peroxidase isoenzyme accumulation was observed. In comparison with the sepals or TNV-infected cotyledons the relative abundance of Prx1 and/or Prx2 and Prx3 represents about one fourth and one seventh, respectively. The highest degree of diversity in distribution of stress-related peroxidase isoenzymes was detected in pistil. From three stress-related peroxidases studied the Prx3 was the only isoenzyme expressed in pistil. This isoenzyme was accumulated in low abundance and reached at about one third of that, present in TNV-infected cotyledons.

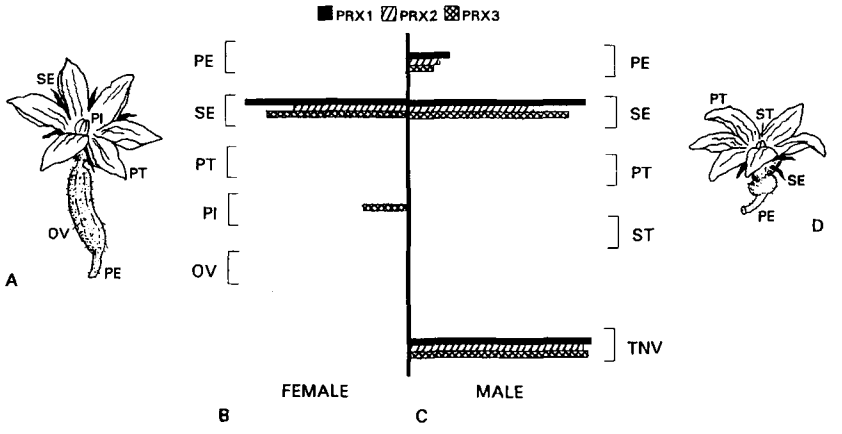

Fig. 3. Distribution analysis of individual stress-related peroxidases in female (*A, B*) and male (*C, D*) flower organs. The bar heights are proportional to the areas of the peroxidase protein bands in Fig. 2*A* resulting from densitometric scanning and computer analysis. PE - pedicel; SE - sepal; PT - petal; PI - pistil; OV - ovary; ST - stamen; TNV - tobacco necrosis virus.

Discussion

The present study has been undertaken to extend our recent reports on cucumber PR-proteins and among them on three anionic peroxidases which are rapidly accumulated during a hypersensitive response (Repka *et al.* 1993, Repka and Slováková 1994) or a stress application (Repka and Vanek 1993).

Peroxidase appears to influence a plethora of physiological processes (Penel *et al.* 1992). However, there is no report on dual function of the same isoenzymes in both defense response and plant development. In this work we observed an organ-specific accumulation of the stress-related anionic peroxidases in a pathogen-independent inductive pathway. In this respect, it is interesting that a similar intrinsic dual functionality has been declared for other groups of defence-related proteins, especially for glycosidases (Lotan *et al.* 1989) and proteinase inhibitors (Keil *et al.* 1989).

In comparison with the measurement of the total soluble peroxidase activity in different organs of male and female flowers, resolution of corresponding extracts in the anodic PAGE system reveals that no peroxidase activity was present in stamen, male and female petals, as well as in ovary. This fact indicates that peroxidase activity identified for these organs must be cathodic. The absence of an anodic peroxidase, especially in stamen, is surprising due to the fact that this type of peroxidase isoenzyme has been considered as a specific and general biochemical marker of stamen morphogenesis in higher plants (Kahlem 1975).

With the aid of immunoblotting experiments and comparative densitometric scannings, a distinct patterns of expression were observed among different flower organs. Unlike male pedicels, no comparable pattern was observed in the female counterpart. The presence of the stress-related peroxidase isoenzymes exclusively in the male organ may be in part related to their potential role in the control of auxin (IAA) catabolism. This statement is strongly supported by the previous conclusion that exogenous auxin can feminize cucumber plants by shortening the male and mixed phase (Frankel and Galun 1977). Although catabolic role was originally suggested for basic peroxidases (Chibbar and Van Huystee 1984, Greppin *et al.* 1986), recent findings have shown that IAA degradation is rather depend on the subcellular localization of isoperoxidases or substrate and cofactor availability (Medina *et al.* 1993, Bernal *et al.* 1993). In this context, we have previously shown that the stress-related anionic peroxidase isoenzymes are located in the cell wall space (Repka *et al.* 1993). It is in this space that auxin catabolism predominantly appears to take place (Ros Barceló and Muñoz 1992). Alternatively, the expression of anodic isoperoxidases in male pedicels may be associated with the ethylene-induced senescence, since the Prx1, Prx2 and Prx3 cucumber isoperoxidases are dramatically induced by ethephon (an ethylene releasing compound, Repka and Vanek 1993). On the other hand, the senescence process in female pedicels must be blocked because this organ represents the only connection between the source of nutrition and developing fruit.

Accumulation of the stress-related peroxidase isoenzymes in both male and female sepals is not clear, but it seems possible that likewise in the male pedicel it associates

with senescence. At the time of anthesis the sepals lost their protective function in developing flower buds. Similarly, in *Nicotiana tabacum* plants, a polypeptide that reacted to PR-1 antiserum appeared in sepal tissue at anthesis (Lotan *et al.* 1989).

We have observed that one of the stress-related peroxidases, Prx3, is specifically expressed in pistil. The function of this isoenzyme in pistil is not known. One possible explanation for the presence of the Prx3 in pistil tissue could be the possibility of a cryptic, nonsymptomatic, infective process being present as a consequence of flower opening and exposure to the surrounding environment. Thus, such situation may mimic a pathogen response. To corroborate this theory, the examination of the temporal mode of Prx3 protein accumulation in developing flower buds must be performed. Another potential function of the Prx3 in pistil may reflect the ability of an anionic peroxidase to change the properties of the extracellular matrix. In the extracellular matrix of pistil of many angiosperm species, a set of glycoproteins (PRPs, HPRGs, PELPs and AGPs) is present (Hong *et al.* 1989, Ye and Varner 1991, Goldman *et al.* 1992, Bacic *et al.* 1988). Among them, in respect to anionic peroxidase, the extensins are the proteins of interest. Thus, expression of the Prx3 isoenzyme may coincide with cross-linking of extensin or extensin-like polymers. Furthermore, we have identified in this study a pistil-specific peroxidase isoenzyme that is not induced by stress but is serologically related to either of the three stress-related peroxidases. Coexpression of these peroxidase isoenzymes in pistil tissue may argue for possible cooperation in the same process. Conversely, other pistil-specific functions of these anionic peroxidases cannot be excluded.

In conclusion, these results show that the stress-related anionic peroxidases exhibit a complex pattern of organ-specific expression. As one approach to assess the detailed role(s) of the individual stress-related peroxidase isoenzymes, studies on the *in situ* localization are in progress.

References

Bacic, A., Gell, A.C., Clarke, A.E.: Arabinogalactan proteins from stigmas of *Nicotiana alata*. - Phytochemistry **27**: 679-684, 1988.

Bernal, M.A., Pedreño, M.A., Calderón, A.A., Muñoz, R., Ros Barceló, A., Merino de Cáceres, F.: The subcellular localization of isoperoxidases in *Capsicum annum* leaves and their different expression in vegetative and flowering plants. - Ann. Bot. **72**: 415-421, 1993.

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chibbar, R.N., Van Huystee, R.B.: Characterization of peroxidase in plants. - Plant Physiol. **75**: 956 - 958, 1984.

Dalet, F., Cornu, D.: Lignification level and peroxidase activity during *in vitro* rooting of *Prunus avium*. - Can. J. Bot. **67**: 2182-2186, 1989.

Dunford, H.B.: Horseradish peroxidase: structure and kinetic properties. - In: Everse, J., Grisham, M.B. (ed.): Peroxidase Chemistry and Biology. Vol. II. Pp. 1-24. CRC Press, Boca Raton 1991.

Felix, G., Meins, F.: Developmental and hormonal regulation of β-1,3-glucanase in tobacco. - Planta **167**: 206-211, 1986.

Frankel, R., Galun, E.: Pollination Mechanisms, Reproduction, and Plant Breeding. - Springer-Verlag, Heidelberg - Berlin - New York 1977.

Frič, F., Fuchs, W.H.: Veräderungen der Aktivität einiger Enzyme in Weizenblatt in Abhängigkeit von der temperaturlabilen Verträglichkeit für *Puccinia graminis tritici*. - Phytopathol. Z. **67**: 161-174, 1970.

Gaspar, T., Penel, C., Thorpe, T., Greppin, H.: Peroxidases: A Survey of Their Biochemical and Physiological Roles in Higher Plants. - University of Geneva, Geneva 1982.

Gaspar, T., Kevers, C., Hausman, J.F., Berthon, J.Y., Ripetti, V.: Practical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. - Agronomie **12**: 757-765, 1992.

Goldman, M.H.de S., Pezzotti, M., Seurinck, J., Mariani, C.: Developmental expression of tobacco pistil-specific genes encoding novel extensin-like proteins. - Plant Cell **4**: 1041-1051, 1992.

Greppin, H., Penel, C., Gaspar, T.: Molecular and Physiological Aspects of Plant Peroxidases. - University of Geneva, Geneva 1986.

Hirsch, A.M., Fortune, D.: Peroxidase activity and isoperoxidase composition in cultured stem tissue, callus and cell suspensions of *Actinidia chinensis*. - Z. Pflanzenphysiol. **113**: 129-139, 1984.

Hong, J.C., Nagao, R.T., Key, J.L.: Developmentally regulated expression of soybean proline-rich cell wall protein genes. - Plant Cell **1**: 937-943, 1989.

Kahlem, G.: A specific and general biochemical marker of stamen morphogenesis in higher plants: Anodic peroxidases. - Z. Pflanzenphysiol. **76**: 80-85, 1975.

Keil, M., Sanchez-Serrano, J., Willmitzer, L.: Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. - EMBO J. **8**: 1323-1330, 1989.

Lotan, T., Ori, N., Fluhr, R.: Pathogenesis-related proteins are developmentally regulated in tobacco flowers. - Plant Cell **1**: 881-887, 1989.

Medina, M.I., De Forchetti, S.M., Tigier, H.A.: Kinetic properties of alfalfa root isoperoxidases with IAA oxidase and syringaldazine oxidase activities. - In: Welinder, K.G., Rasmussen, S.K., Penel, C., Greppin, H. (ed.): Plant Peroxidases. Biochemistry and Physiology. Pp. 175-180. University of Geneva, Geneva 1993.

Memelink, J., Linthorst, H.J., Schilperoort, R.A., Hoge, H.J.C.: Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. - Plant mol. Biol. **14**: 119-126, 1990.

Nakanishi, F., Fujii, T.: Appearance of peroxidase isozymes in floral-initiated shoot apices of *Pharbitis nil*. - Physiol. Plant. **86**: 197-201, 1992.

Penel, C., Gaspar, T., Greppin, H.: Plant peroxidases 1980 - 1990. Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspects. - University of Geneva, Geneva 1992.

Repka, V., Kollerová, E., Slovák, M.: The extracellular acidic and basic virus-elicited proteins of cucumber cotyledons. - Biol. Plant. **35**: 87-93, 1993.

Repka, V., Slováková, Ľ.: Purification, characterization and accumulation of three virus-induced cucumber peroxidases. - Biol. Plant. **36**: 121-132, 1994.

Repka, V., Slováková, Ľ., Kollerová, E., Slovák, M.: [Association of the enzymatic activity with expression of PR-proteins in leaves of *Cucumis sativus* L. infected by tobacco necrosis virus.] - In: X. National Genetic Conference. Pp. 191-192. Agricultural University, Brno 1991. [In Slovak]

Repka, V., Vanek, G.: Characterization of the stress-related, extracellular anionic peroxidases from cucumber. - In: Welinder, K.G., Rasmussen, S.K., Penel, C., Greppin, H. (ed.). Plant Peroxidases: Biochemistry and Physiology. Pp. 355-360. University of Geneva, Geneva 1993.

Ros Barceló, A., Muñoz, R.: Peroxidases: Their role in the control of plant cell growth. - In: Greppin, H., Penel, C., Gaspar, T. (ed.): Peroxidases 1980 - 1990. Progress and Prospects in Biochemistry and Physiology. Pp. 71-89. University of Geneva, Geneva 1992.

Van Huystee, R.B.: Some molecular aspects of plant peroxidase biosynthetic studies. - Annu. Rev. Plant Physiol. **38**: 205-217, 1987.

Welinder, K.G., Rasmussen, S.K., Penel, C., Greppin, H.: Plant Peroxidases: Biochemistry and Physiology. - University of Geneva, Geneva 1993.

Ye, Z. -H., Varner, J.E.: Tissue-specific expression of cell wall proteins in developing soybean tissues. - Plant Cell **3**: 23-37, 1991.

Zhou, X., Han, Y., Yang, W., Xi, T.: Somatic embryogenesis and analysis of peroxidase in cultured lettuce (*Lactuca sativa* L.) cotyledons. - Ann. Bot. **69**: 97-100, 1992.