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Expression of cucumber stress-related anionic peroxidases during flower development or a cryptic infective process

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Abstract

The striking diversity in the expression pattern of the stress-related anionic peroxidase was observed during development of female cucumber flower. While the isoenzyme Prx3 was accumulated constitutively in the course of flower development, the expression patterns of other two isoenzymes (Prx1 and Prx2) were restricted to the period after flower opening. The virus infection was simulated by careful opening of the intact female flower buds 3 d before anthesis followed by exposition to the glasshouse environment for 3 d. The results obtained in this experiment revealed a marked accumulation of the isoenzyme Prx1 and Prx2 at anthesis. Under normal flower development, the pistils did not accumulate these isoenzymes at this stage. In contrast, the pattern of expression of Prx3 as well as of the pistil-specific peroxidase isoenzyme remained unchanged, confirming a constitutive type of expression. Beside the pistil, a 3-d exposition of the stripped flowers resulted in a marked accumulation of Prx1 and Prx2 isoenzymes also in both adjacent flower organs - the ovary and the pedicel. At the same time of the normal development of female flower these organs did not accumulate these isoenzymes.

Additional key words: *Cucumis*, electrophoresis, PR-proteins, tobacco necrosis virus, Western blotting.

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Abbreviations: BSA - bovine serum albumine; CSPD-disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1.^{3,7}] decan}-4-yl) phenyl phosphate; DEA - diethanolamine; ECL - enhanced chemiluminiscence; EDTA - ethylene- diaminetetraacetic acid; HPRGs - hydroxyproline-rich glycoproteins (extensins); PAGE - polyacrylamide gel electrophoresis; PR - pathogenesis-related; PVDF - polyvinylidene difluoride; PVP - polyvinylpyrrolidone; SIBA - slot immuno binding assay; TNV - tobacco necrosis virus.

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This paper is dedicated to the memory of Doc. Ing. Fridrich Frič, DrSc., an outstanding scientist and great colleague, who died suddenly at the high point of his successful research career.

Introduction

The plant peroxidase system contains a set of peroxidase isoenzymes which carry out a series of biosynthetic and degradative functions in the normal developmental programme or different stress situations. Although the enzyme has been known for many decades (Saunders *et al.* 1964), its actual role(s) remains ambiguous to date. Leaving aside the widely observed catalytic properties (Berlin and Barz 1975, Gaspar *et al.* 1985, Perrey *et al.* 1989, Medina *et al.* 1993), the main problem in defining specific functions of plant peroxidases is the existence of both many of potential substrates and a multitude of isoenzyme forms. Last but not least, the precise subcellular localization of the particular peroxidases in plant cells has been in almost all cases ignored. There is a continuously growing knowledge about the expression of plant peroxidases as a response to environmental stimuli *(e.g.* pathogenesis, wounding, light, temperature *etc.*). Recently it has been demonstrated that peroxidase, as a member of the antioxidant defense system, plays an active role in plant hypersensitive disease resistance response (Levine *et al.*1994), probably by blocking the programmed cell death (Hockenbery *et al.* 1993). On the other hand, there is little information available concerning the expression of peroxidase isoenzymes in the plant development (Hendriks and Van Loon 1990, Zheng and Van Huystee 1992, Innocenti *et al.* 1994). Although the fast-moving set of the stress-related anionic peroxidases of cucumber was primarily observed in cotyledons reacting hypersensitively to TNV infection (Repka *et al.* 1993a), in our recent paper we have shown that these isoenzymes are differentially expressed in developing male or female flowers in an organ specific manner. Especially for female flowers a unique pattern of expression was documented (Repka and Jung 1995). Due to the fact that this pattern of expression was observed at anthesis period we could not exclude that this was the result of a cryptic, nonsymptomatic, infective process after flower opening and exposure to the environment. These results prompted us to further investigate the temporal mode of the stress-related peroxidases accumulation in developing female cucumber flowers using polyclonal antibodies and immunoblotting technique.

Materials and methods

Plant material: Cucumber seeds (*Cucumis sativus* L. *cv.* Laura) were surface sterilized with 0.8 % *Domestos* (*Unilever Ltd.*, Bratislava, Slovakia) and grown until flowering (*ca.* two months) in sterilized soil in a glasshouse (temperature 20 - 32°C). Plants were watered to saturation daily and fertilized weekly with *OBM* fertilizer or with *Substral* (*Henkel AS*, Bratislava, Slovakia).

Inoculation of plants: Cotyledons of 7-d-old plants were abrased and infected with TNV as described in Repka and Slováková (1994). Control plants were treated similarly with virus-isolation buffer alone.

Flower sorting and manipulation: To analyse temporal mode of the peroxidase expression, female flowers were harvested at respective stages and separated into major organs. In other case, corollas were carefully dissected from flower buds at the relevant developmental stages with a razor blade without any damage to the pistil tissue. The plants with stripped flowers were exposed to glasshouse environment at different intervals and afterwards immediately processed.

Protein extraction: To extract total water-soluble proteins, flower tissues were ground in a prechilled mortar at 4 °C in appropriate volumes of TRISEPAC buffer (50 mM TRIS-HCl, pH 8.0, 500 mM saccharose, 1 mM EDTA, 0.2 % insoluble PVP, 6 mM ascorbic acid and 0.1 % cysteine). The homogenate was filtered through two layers of gauze and the filtrate was centrifuged for 20 min at 15 000 *g* at 4 °C. Supernatants were used immediatelly or kept frozen at -20 °C for future use.

Determination of peroxidase activity and protein content: Peroxidase activity was determined at 20 °C in the presence of 0.3 % hydrogen peroxide and 0.3 % guaiacol in 50 mM sodium acetate buffer, pH 5.2, and is expressed as a change in the absorbance at 470 nm [ΔA_{470} mg^{-1}(protein) s^{-1}]. Protein concentration in the homogenates was determined colorimetrically according to Bradford (1976) with BSA as the standard.

Gel electrophoresis (PAGE) and staining of peroxidase isoenzymes: The anionic peroxidases were separated in 10 % native PAGE gels as described previously (Repka and Slováková 1994). Peroxidase isoenzymes on gels were visualized by activity staining with 3-amino-9-ethylcarbazole (*Amresco*, Solon, USA) and H_2O_2 (0.04 % and 0.03 %, respectively) in 50 mM sodium acetate buffer at pH 5.2.

Immunoblotting (Western blot): Proteins separated on native PAGE gels were electro-transferred for 20 h at 50 mA (4 °C) to nitrocellulose membranes (*Protran BA-85*, 0.45 mm, *Schleicher & Schuell*, Dassell, Germany) in a blot buffer containing 0.7 % acetic acid. Blots obtained from native gels were heated at 80 °C for at least 6 h to inactivate endogenous peroxidase activities. The blots were then blocked for 1 h in 5 % non-fat dry milk (*Blotto*), and subsequently processed as described previously (Repka and Slováková 1994). To obtain a complex expression pattern of the stress-related peroxidases, a mixture of cucumber anti-RX 1, 2 and 3 antibodies was employed.

SIBA-ECL: The low-level expression of the PR-gene products was estimated with the aid of Slot Immuno Binding Assay coupled with enhanced chemiluminiscence detection (*SIBA-ECL*). Individual samples equivalent to 10, 20, 40 and 60 μg of total proteins were slotted onto PVDF membrane using the Slot Blot apparatus (*PR 648, Hoefer Scientific Instruments*, San Francisco, USA). PVDF membrane (*Hybond-PVDF*, 0.45 μm, *Amersham International*, Buckinghamshire, UK) was sequentially soaked in methanol, sterile distilled water and TEN buffer (50 mM TRIS-HCl, pH 7.4, 5mM EDTA, 150 mM NaCl) for 1, 5 and 15 min, respectively. After loading the samples, the membrane was blocked for 1 h at room temperature in

5 % *Blotto*. Following the overnight incubation of membrane with respective primary antibody at 4 °C, the membrane was washed four times in TEN buffer + 0.1 % Tween 20 for 10 min each. Goat antirabbit IgG alkaline phosphatase conjugate at 1:1000 dilution (*SANOFI Diagnostics Pasteur*, Marnes la Coquette, France) was used as secondary antibody for 2 h at room temperature. The second washing cycle in TEN + 0.1 % Tween 20 for 2 × 10 and 2 × 15 min was carried out to remove unbound antibody. This was followed by washing in *DEA* substrate buffer (pH 10, 1mM $MgCl_2$, *TROPIX*, Bedfrod, USA). Then the membrane was sealed in a hybridization bag and incubated in a small volume *CSPD* chemiluminiscence solution (*Boehringer Mannheim*, Wien, Austria) for 10 min. This wet membrane was incubated for 35 min at 37 °C between two sheets of overhead transparency film (*Minolta*, Bratislava, Slovakia) to activate the chemiluminiscent substrate.The damp membrane was then sandwiched between two flat sheets of *Frischhalte* folies (*ALU - FIX*^{TM}, Neudorf, Austria) and put in a cassette with *Hyperfilm*^{TM}*-ECL* film (*Amersham International*, Buckinghamshire, UK) and two *Cronex* intensifying screens (*Du PONT Co.*, Willmington, USA). For maximum sensitivity and accurate quantitation of results the *Hyperfilm*^{TM}*-ECL* film was preexposed to an instantaneous flash of light using the *Sensitize*^{TM} pre-flash unit (*Amersham International*, Buckinghamshire, UK). The films were exposed for varying durations of time and processed using standard X-ray developer and fixer.

Reprobing of PVDF membrane: For screening the presence of different antigens on the same PVDF membrane this was reprobed as recommended in the *Hybond-PVDF* stripping protocol (*Amersham International*, Buckinghamshire, UK). Four different anti-PR-proteins sera were tested (anti PR-1, PR-2, PR-3 and HPRG). After stripping of the antibody from the membrane, this was blocked and immunoprocessed according to the above mentioned procedure.

Results

Stress-related peroxidase isoenzymes are temporally regulated during pistil development: To provide reference points for gene expression events during female flower development we divided it into 7 stages (from +3 to -3) as related to anthesis (stage 0). Each stage represents a 24 h increment as related to anthesis. The particular stages of female flower development were discriminated on the basis of length, shape and coloration of petal tissue. Due to the fact that from stage +1 the length of the corolla did not change significantly, the coloration pattern of petal tissue proved to be the best discrimination marker:

Stage –3: Petals fused completely at their margins; corolla pale green.
Stage –2: Calyx bulge enlarging horizontally and cup-shaped; corolla yellow-green.
Stage –1: Corolla elongating rapidly; petal tips slightly open; petals pale yellow.
Stage 0: Flower opens; corolla limb fully expanded and bright yellow.
Stage +1: Flower begins to close; corolla limb bright orange.
Stage +2: Corolla limb halfway closes; corolla pale orange.
Stage +3: Corolla closes; petal tips curled; corolla limb light orange.

The highest peroxidase activity was observed three days before anthesis (stage -3) and it represented at about fourfold of that in TNV-infected cotyledons (Fig. 1*A*). However, in the six consecutive days, the total peroxidase activity decreased dramatically and the lowest activity was determined at stage +3. An inverse time-course pattern was observed for accumulation of an anodic peroxidase (Fig. 1*B*). The stress-related peroxidase isoenzymes (Prx1 and Prx2) together with a pistil-specific isoenzyme represented virtually all the anionic peroxidase activity presented in the extracts. Subsequently, two different expression patterns were typical for particular isoenzymes. The isoenzyme Prx3 as well as the pistil-specific one were coordinately and constitutively expressed from the stage -3 to stage +3, although the quantitative differences among the particular developmental stages were evident. The protein gel immunoblot (Fig. 1*C*) reflects the pattern of the activity stained gel (Fig. 1*B*). The

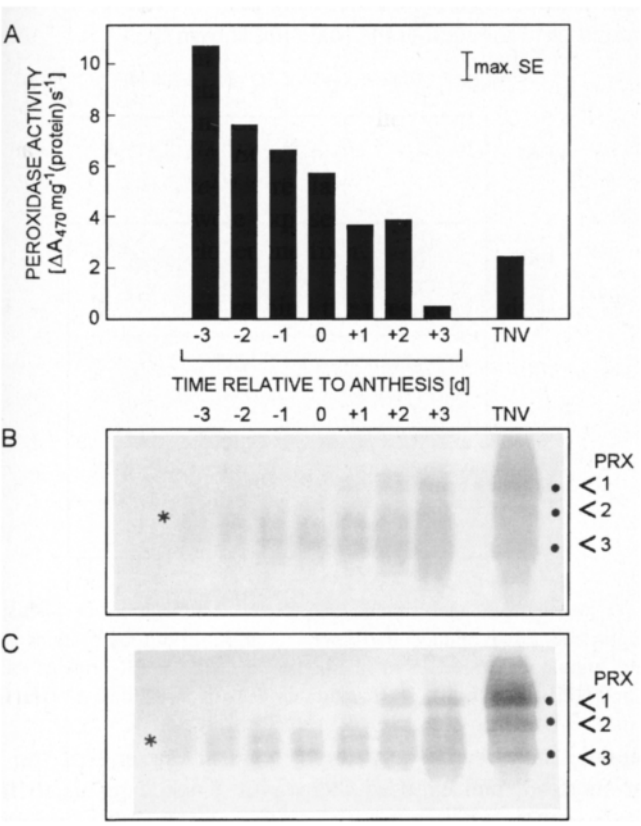

Fig. 1. Time-course of soluble peroxidase activity in extracts from pistils during female flower development. *A*. The total peroxidase activity in the respective days of development. Values represent the means of three separate measurements. Electrophoretic patterns of anionic peroxidases in pistil extracts stained for peroxidase activity using 3-amino-9-ethylcarbazole (*B*) and corresponding Western blot analysis (*C*) using the antiserum against purified cucumber peroxidase. Each lane was loaded with 25 mg and 100 mg of proteins for activity staining and immunoblotting, respectively. Small *asterisk* in *B* and *C* denotes pistil-specific peroxidase isoenzyme.

time-course expression of the isoenzymes Prx1 and Prx2 was restricted exclusively to post-anthesis period (stage +1 to +3). A maximum rate of accumulation for both isoenzymes was reached at stage +3.

Accumulation of the stress-related peroxidase isoenzymes as the consequence of a cryptic infective process: With the aid of very sensitive SIBA-ECL technique the expression patterns of four pathogenesis-associated products were followed in respective pistil extracts. Immunoblotting of total proteins extracted from pistils at particular days (stages -3 to +3) with respective antisera revealed the immunopositive signal only for PR2 (glucanase) protein and PR3 (chitinase) protein (Fig. 2). Both antigens were barely detectable on the first day after flower opening but signals increased gradually in the forthcoming days. The anti-PR1 as well as the anti-HPRG sera did not cross-reacted with the extract from pistils, even though the excess of the protein was applied onto the membrane (data not shown).

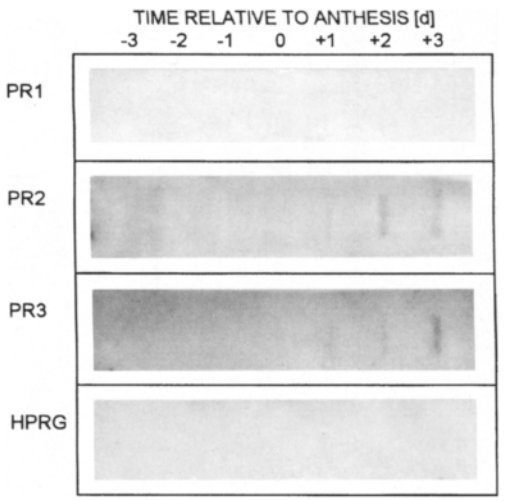

Fig. 2. SIBA-ECL immunoblot analysis of PR-protein accumulation in pistils during normal developmental programme. Equal aliquots corresponding to 40 μg of total proteins were applied onto the membrane and immunoprocessed using antibodies specific to PR-proteins of type 1 (PR1), 2 (PR2), 3 (PR3) and HPRG (extensin). The membranes were exposed to X-ray film for 1 h.

We also simulate an infective process by careful opening of the intact female flower buds (at stage -3) and exposed them with a naked pistils to the glasshouse environment for next 3 d. The analysis of the total soluble peroxidase activity showed that rate of accumulation in unexposed pistils has decreasing tendency towards the anthesis while in those exposed was dramatically increasing (Fig. 3*A*). The electrophoretical separation of the pistil extracts from both unexposed and exposed flowers (Fig. 3*B*) showed a marked difference particularly in comparison with pattern from pistil extracted at anthesis (stage 0). The isoenzyme Prx1 and in a lesser extent the Prx2, respectively, were highly or moderately accumulated exclusively in exposed pistils. The Western blotting also confirmed these results even

though the immunode- tection of the Prx2 isoenzyme was just at the limit of immunoblot analysis (Fig. 3*C*). In the pistil extracts from unopened flower buds neither activity staining nor Western blotting revealed the presence of either of the two peroxidase isoenzymes.

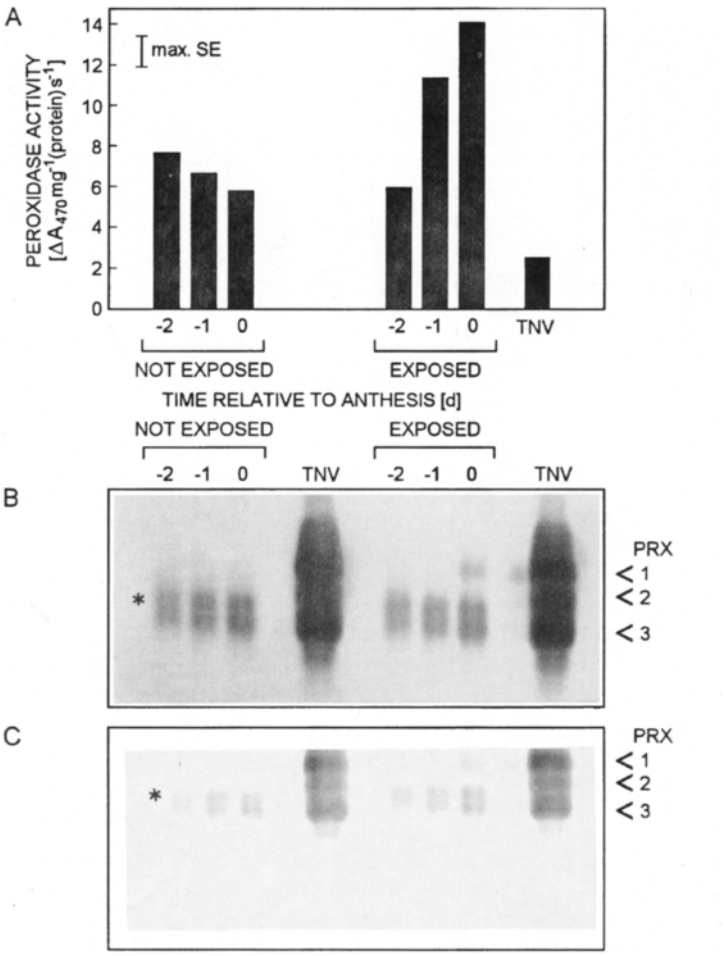

Fig. 3. Effect of a 3-d exposure of stripped female flowers on soluble peroxidase accumulation in pistil. The total peroxidase activity in unexposed and exposed pistil extracts (*A*). Values are means of three separate measurements. Comparative PAGE under native conditions. Gels run at 4 °C were stained for anionic peroxidase activity (*B*) or subjected to Western blotting and immunodecoration with specific antiserum raised against purified cucumber peroxidases (*C*). *Asterisk* denotes a novel pistil-specific peroxidase isoenzyme. Nex - not exposed; Ex - exposed.

The intact female flowers opened at stage -3 and exposed for three consecutive days to the glasshouse environment showed a dramatical increase of the total soluble peroxidase activity also in two flower organs adjacent to the pistil (*i.e.* ovary and pedicel). The highest rate of the total peroxidase accumulation was observed 3 d after

pistil exposure (Fig. 4). Activity stained gel also revealed that beside the pattern typical for exposed pistil, stress-related peroxidase isoenzymes were differentially accumulated in both ovary and pedicel (Fig. 4*B*). Western blotting analysis

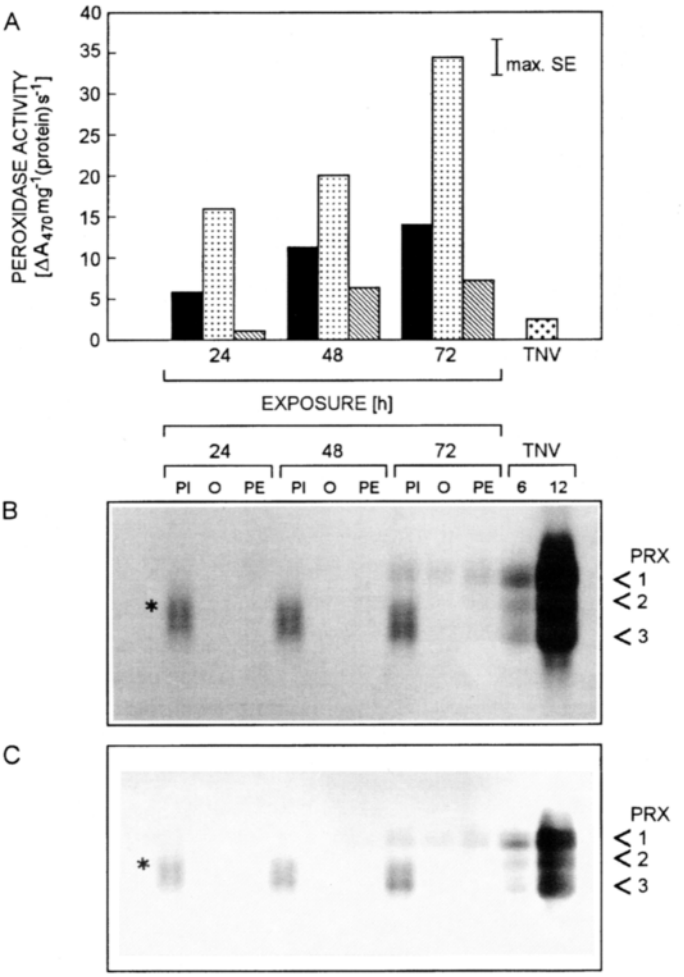

Fig. 4. Effect of a 3-d glasshouse exposure of the stripped female flowers on accumulation of the stress-related anionic peroxidases in pistil and adjacent flower organs. *A* represents the total soluble peroxidase accumulation in different flower organs (*full columns* - pistil, *dotted columns* - ovary, *hatched columns* - pedicel). The values are means of three separate measurements. Gel electrophoretical analysis of time-course of the anionic peroxidase isoenzymes in three flower organs as result of the flower exposure to glasshouse environment. The lanes marked as *PI* (pistil), *O* (ovary) and *PE* (pedicel) were loaded with 25 mg of total proteins. The lanes of TNV-infected extracts denoted as *6* and *12* were loaded with 6 mg and 12 mg of total proteins, respectively. The comparative gel (*C*) equivalent to *B* was subjected to immunoblotting using the antiserum against the purified peroxidases. *Asterisk* denotes pistil-specific peroxidase isoenzyme.

confirmed that the Prx1 was the only isoenzyme accumulated in ovary tissue while all three isoenzymes, although with a differential rate, were accumulated in the pedicel (Fig. 4*C*).

Discussion

We have recently reported the detection and spatial accumulation of the stress-related anionic peroxidase in female cucumber flowers (Repka and Jung 1995). The pistil was the only flower organ with the highest degree of diversity in distribution of the stress-related peroxidase isoenzymes. Accumulation of these isoenzymes at anthesis period automatically raised the question whether it is a result of normal developmental programme or the consequence of a cryptic, nonsymptomatic, infective process resulting from flower opening and exposure to the environment. Therefore, to confirm this working hypothesis we have extended our previous observations to show a temporal accumulation of the stress-related peroxidase in a developmentally dependent manner. The electrophoretical and immunochemical analysis of this temporal mode of accumulation during pistil development has revealed an unexpected but interesting patterns of expression. While the isoenzyme Prx3 was constitutively coexpressed with the pistil-specific peroxidase during development, other two isoenzymes studied (Prx1 and Prx 2) were accumulated exclusively at period after flower opening. It is important to note that the constitutive type of Prx3 isoenzyme expression may be restricted only to the pistil since according to our previous observations (Repka and Vanek 1993) it was always accumulated as a response to primary induction by different stimuli (*e.g.* physical, chemical, *etc.*). There is a reasonable assumption that a constitutive expression of the Prx3 during the pistil developmental programme may argue for a very important function in this organ. However, this activity probably does not associate with the best-characterized structural cell wall protein-extensin (HPRG) since as revealed by Northern blot analysis, its gene expression is much higher in roots and stems (Showalter and Varner 1987, Gatehouse *et al.* 1990, Showalter *et al.* 1991) than in leaves and flowers. These results are consistent with the recent data of Tiré *et al.* (1994), although they detected a low-level accumulation of the extensin transcript also in flower bud of *Nicotiana plumbaginifolia.* In addition, our results demonstrating the absence of extensin in stripped female flowers (Fig. 2) also argue against possible association of the Prx3 isoenzyme with this type of structural protein functioning in the rigidification of cell wall structures (Fry 1986). Such differential patterns of expression may probably underline the existence of an intrinsic dual functionality of some of the stress-related anionic peroxidase isoenzymes.

To give a better support to our hypothesis that a cryptic infective process being present as a result of the flower opening to surrounding environment, experiment was designed to investigate the time-course accumulation of other endogenous PR-gene products. The pistil extracts corresponding to respective days of development were screened by using of four different antisera with well defined specificity. The absence of PR1-like antigen was not surprising since this type of protein was present

neither healthy nor TNV-infected cucumber cotyledons (unpublished results). On the other hand, the absence of HPRG signal is unexpected due to the fact, that these molecules, localized in the primary cell wall have been implicated in plant defense (Showalter and Varner 1987, Showalter and Rumeau 1990). Alternatively, the absence of the signal could be explained by absence of the protein. This fact is strongly supported by the findings of Mouly *et al.* (1992) on sunflower plants infected with *Sclerotinia sclerotiorum* which started massive accumulation of the HPRG transcript just 3-d after infection.

Two other PR-proteins (PR2 and PR3) gave highly positive immunoreactive signals, just at the period when flowers became fully opened (stage +1 to +3). In contrast to our results, Lotan *et al.* (1989) similarly studied the expression of the PR2 protein in developing tobacco flowers. However, following examination of the temporal mode of PR2, N, O-like protein accumulation they eliminated the possibility of a cryptic infective process. It was mainly due to the fact that immunoreactive signal was already detected in unopened flower buds (*i.e.* about 2 d before anthesis) and increased markedly at the time of anthesis. In this context, we consider their result not fully contradictory to our ones since the PR2 family represents a group of serologically-related proteins. Therefore, we cannot exclude the possibility that in this case the pathogen-induced and developmentally regulated signals overlapped each other.

Plants grown under glasshouse conditions are exposed to diverse stress factors of unknown origin (Negaard and Hoffmann 1989). Recently, Jung *et al.* (1995) have shown that glasshouse-grown sunflower plants produced high level of PR-proteins as compared with an environmental chamber-grown ones. From this point of view, the glasshouse exposure seemed to be a very good tool to provoke infection process. Exposure of stripped cucumber flowers to such environment resulted in a clearly visible accumulation of the Prx1 and 2 isoenzymes strictly on the third day. It is noteworthy, that such type of expression pattern for these isoenzymes copy that of TNV-infected cotyledons (Repka and Vanek 1993). At the same time, this expression pattern was not restricted only for pistil but these isoenzymes were also accumulated in adjacent flower organs. The question may arise whether such accumulation could be a consequence of wounding. It is well documented that wounding is frequently accompanied by the changes in cell properties (Davies and Schuster 1981, Walker-Simmons *et al.* 1984). However, our previous results regarding the wounding response in cucumber argue against this possibility. Firstly, wounding response in cucumber cotyledons represents a very rapid process and different PR-protein start to accumulate already 1 d after wounding (Repka and Vanek 1993). Secondly, pistil extirpation from intact cucumber flowers resulted only in a transient accumulation of stress-related peroxidase followed by a marked decreasing on the fourth day (unpublished results). This is not a typical wounding response in cucumber since in that case the massive accumulation persisted 8 d after wounding (Repka *et al.* 1993b).

The results presented in this work provide the first evidence for functional diversity of the stress-related anionic peroxidase isoenzymes primarily identified in disease response.

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