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Serologically-related anionic peroxidases from petunia and cucumber can substitute flavonoid antioxidants

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Abstract

In this study we have investigated whether naturally occurring flavonoid-deficient mutant Red Star of *Petunia hybrida* is capable of metabolizing H_2O_2 by invoking other antioxidant enzyme system. We demonstrated that reduced flower pigmentation due to a reduction in the chalcone synthase mRNA expression results in strong H_2O_2 accumulation accompanied by the induction of a specific set of anionic peroxidase (PRX), serologically-related to main cucumber srPRX. We found correlation between rate of H_2O_2 accumulation and qualitative, as well as quantitative changes in the srPRX expression which seems to be determined by flower phenotype. In detached flower buds cultured *in vitro* both abscisic acid and anther extirpation prevented anthocyanin pigmentation, and thus flavonoid biosynthesis, resulting in a marked accumulation of immunoprecipitable srPRX. In contrast, pigmented flowers cultivated under the same conditions did not accumulate corresponding srPRX. The results suggest that a specific set of anionic PRX can substitute for the absence of flavonoid antioxidants.

Additional key words: abscisic acid, chalcone synthase, *Cucumis*, electrophoresis, immunoblotting, *in vitro* culture, magnetic immunoprecipitation.

Introduction

Even under optimal conditions many processes in metabolically active cells produce reactive oxygen species (ROS): singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radical. As an example, the photosynthetic electron transport system represents the major source of these ROS (Asada 1994). Besides these non-stressful conditions, a man-made stresses (*e.g.* accumulation of the air pollutants such as ozone and sulfur dioxide, certain herbicides, *etc.*) provoke increased production of ROS. To cope with the toxicity of ROS a highly efficient antioxidative defense system, composed of both non-enzymic and enzymic constituents, is evolved in all plant cells. The non-enzymic antioxidants (scavengers and quenchers) generally represent a group of small molecules operating primarily in destruction of hydroxyl radicals and singlet oxygen. These low-molecular secondary products include a great variety of hydrofilic (ascorbate, glutathione) and lipophilic (α-tocopherol, carotenoids) antioxidants, nitrogenous, and sulfur-containing materials (Larson 1995). Moreover, plant-derived polyphenols such as flavonoids have repeatedly been shown to be ones of the most effective antioxidants. Flavonoid compounds having *o*-hydroxylation and/or multiple hydroxylation, such as quercetin and myricetin, have been demonstrated to be particularly effective antioxidants in many studies (Dziedzic and Hudson 1983, Pratt and Hudson 1990).

The enzymic antioxidative components include superoxide dismutase (SOD), catalase (CAT) and PRX whose primarily scavenge superoxide and H_2O_2. In context with the later, H_2O_2 formed either as a by-product of normal metabolism or produced under specific situations when

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Abbreviations: ABA - abscisic acid; BSA - bovine serum albumine; CAT - catalase; CHS - chalcone synthase; EDTA - ethylenediaminetetraacetic acid; NC - nitrocellulose; PAGE - polyacrylamide gel electrophoresis; PBS - phosphate bufferred saline; PRX - peroxidase; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SDS - sodium dodecylsulphate; srPRX - stress-related peroxidase; SOD - superoxide dismutase.

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oxidative burst occurs has been shown to be a key component in many events (Lamb and Dixon 1997). H_2O_2 can easily penetrate cellular membranes and react at sites distant from the site of its formation. If not promptly scavenged, H_2O_2 can participate in ROS interconversions like Fenton reaction generating the very reactive hydroxyl radical (Halliwell 1987). Minimizing of harmful reactions from inevitably formed H_2O_2 is possible due to the activity of CAT and PRX.

In every organism there is a dynamic equilibrium between reactions generating ROS, and activity of antioxidants (Gonner and Schlosser 1993, Levine *et al.* 1994, El-Zahaby *et al.* 1995, Fodor *et al.* 1997, Vanacker *et al.* 1998). In contrast to this coordinated action, little is known about the response when one of these counterparts is blocked in the activity or is completely missing. In addition, there is no report whether various members of cellular antioxidant defense system could substitute each other. Therefore, in an attempt to evaluate and clarify this potential we have used naturally occurring *P. hybrida* flavonoid-deficient (CHS) mutant Red Star and serological cross-reactivity between petunia and cucumber anionic PRX. Utilizing this model we investigated the impact of absence of flavonoids on the srPRX activity.

Materials and methods

Plants: For all studies we used a natural CHS mutant of *Petunia hybrida* line Red Star (R132). Petunia seeds were first surface sterilized with 0.8 % *Domestos* (*Unilever Ltd.*, Bratislava, Slovakia) and the plants were grown under standard greenhouse conditions (Repka and Fischerová 1997a).

***In vitro* cultivation of flower buds:** To demonstrate the effect of ABA and flower emasculation on the peroxidase accumulation in corolla tissue, young flower buds at developmental stage 3 (Weiss and Halevy 1989, Weiss *et al.* 1992) were used. Intact flowers were cut from the plant approximately 1 cm below the sepals and placed in a *Magenta* container (*Magenta Corp.*, Chicago, USA) containing 20 cm^3 of a 150 mM sucrose (Suc) solution, pH 5.5 (Weiss and Halevy 1989), supplemented with abscisic acid (ABA; *Serva*, Heidelberg, Germany). For some experiments emasculated flower buds were used. In this case, it was necessary to make a small longitudinal incision at the base of the corolla tube through which the anthers were carefully extirped from the flowers buds. Both complete or emasculated flower buds were incubated in a cultivation chamber at 24 °C and constant light (*Cool White* and *Grolux* fluorescent lamps, *Sylvania,* Hartog den Bosch, The Netherlands, irradiance 165 W m^{-2}) for the times indicated in the figures.

Flower sorting and manipulation: To analyze the spatial mode of the peroxidase expression in variable pigmented corollas (red or white pigmented sectors of the corolla), the flowers were sorted on the basis of CHS expressing area quantification (red sectors). The flowers with variegated phenotypes, from completely pigmented (red), with reduced pigmentation to completely unpigmented (white), were first photographed using *LCD Digital Camera QV-10A* (*CASIO Computer Co.*, Tokyo, Japan). Individual pictures (flowers) were then processed using a software *QV-LINK*. Digital images were exported to a *SIGMA Scan* programme (version 3.0, *Jandel Scientific Software*, Erkrath, Germany) and analyzed. Alternatively, the images were videoprinted using *Kodak Digital Science SP700* colour printer (*Eastman Kodak Co.*, Rochester, USA) and prints analyzed planimetrically. The results obtained by the both methods were very similar (difference < 1 %).

Preparation of extracts: Whole flowers were ground in a prechilled mortar and rapidly homogenized at 4 °C using *DIAX 900* tissue grinder (*Heidolph*, Kelheim, Germany) in 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, 0.1 % cysteine). The homogenate was filtered through two layers of *Miracloth* (*Calbiochem AG*, Zug, Switzerland) and the filtrate was centrifuged for 5 min at 20 000 *g* at 4 °C. For some experiments the pigmented parts of the flowers were separated from the white parts before extract preparation which was done as described above.

Sample analysis: Within 10 min after the extraction was completed the rate of hydrogen peroxide accumulation was analyzed using starch/KI reagent (Olson and Varner 1993, Repka and Fischerová 1997b). The formation of the starch-KI complex was measured at 620 nm using *Shimadzu UV-1601* spectrophotometer (*Shimadzu Europe*, Manchester, UK). Supernatant was further concentrated using *Microcon-3* microconcentrators (*Amicon*, Witten, Germany) and kept at -20 °C until further use. Protein concentration was determined colourimetrically according to Bradford (1976) with BSA as a standard. The supernatant containing soluble proteins was used for measurement of PRX activity according to Repka and Slováková (1994).

Analytical PAGE: Proteins were fractionated either by non-denaturing PAGE using a 4 % stacking gel and 10 % separating gel (Repka and Fischerová 1998) or under denaturing conditions as described by Laemmli (1970).

Each lane of the gel was loaded with equal amount of protein and bromophenol blue as marker. Peroxidase isoenzymes on gels were visualized by *in gel* activity staining procedure according to Repka and Fischerová (1997b).

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 0.7 % acetic acid (for non-denaturing gels) or 40 mM phosphate buffer (pH 6.5 for denaturing gels) as a transfer medium. After inactivation of endogenous peroxidases and blocking of the membrane (Repka *et al.* 1996), PRX or CHS protein was detected using anti-PRX or anti-CHS specific antibodies as described before (Repka and Slováková 1994).

Magnetic immunoprecipitation: Immunoprecipitation of the PRX activity was carried out using direct immunomagnetical isolation of specific protein from crude homogenates of petunia flowers. Briefly, the *Dynabeads M-280* Sheep anti-Rabbit IgG (*Dynal A/S*, Oslo, Norway) superparamagnetic beads were washed with PBS/BSA (pH 7.4, phosphate buffered saline, 0.1 % BSA) according to supplier's protocol. Required quantity of *Dynabeads* [10^8 particles cm^{-3}(homogenate)] was pipetted into an Eppendorf tube and 0.01 cm^3 of anti-PRX IgG were added. The mixture was incubated for 24 h at 4 °C using *Dynal MX1* sample mixer, then the beads coated with primary antibody were collected and washed with PBS/BSA using *Dynal Magnetic Particle Concentrator* (*MPC-E-1*). After the last washing the coated *Dynabeads* were added to crude protein homogenates (0.05 cm^3) to a final volume of 0.2 cm^3 with immunoprecipitation buffer PBS/BSA and again incubated at 4 °C for a minimum of 2 h, providing gentle shaking. The beads were collected for 2 min with a *Dynal MPC-E-1* and supernatant discarded. Immunomagnetically isolated complexes were directly resuspended in 0.025 cm^3 of SDS-PAGE loading buffer (Laemmli 1970) and heated to 100 °C for 3 min to elute of proteins from beads. *Dynabeads* were collected at the bottom of the tube using particle concentrator and clear supernatants were directly loaded onto electrophoresis gel (12.5 % SDS-PAGE). Immunoisolated proteins separated by gel electrophoresis were transferred to a NC-membrane and the Western blots probed with an antibody specific for the target protein (PRX) were detected using *Transcend*™ *Chemiluminescent Translation Detection* system (*Promega Corp.*, Madison, USA) and *Hyperfilm*™*-ECL* film (*Amersham International*, Buckinghamshire, UK). The films were sensitized and processed as described in Repka *et al.* (1996).

Results

Differential expression of CHS and PRX in *P. hybrida* CHS mutant: The flowers of a natural *P. hybrida* flavonoid-deficient mutant Red Star (chalcone synthase mutant) exhibit alternating white and red sectors. Western blot analysis of protein isolated from white and red sectors of floral tissue revealed variegated expression of both enzymes studied. The content of chalcon synthase (CHS) protein is strongly reduced in white sector as compared with the red sector (Fig. 1*A*). Unlike to CHS, the PRX protein content in white sector compared with that obtained from red sector remains unaffected.

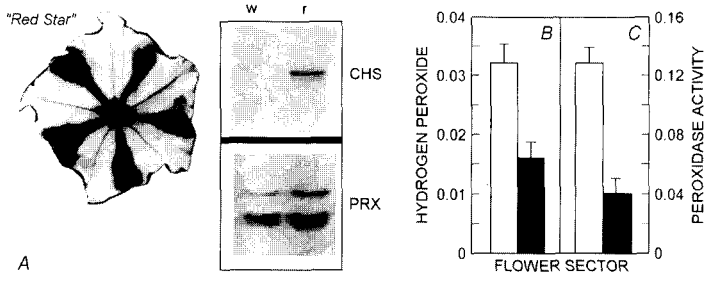

Fig. 1. Comparison of protein, H_2O_2, and specific enzyme activity in pigmented and unpigmented sectors of petunia Red Star mutant flower. *A* - Western blot analysis of 25 μg floral protein extracted from white (w) and red (r) sectors of petunia flower. The blot was probed with CHS and PRX specific antibodies prepared against respective protein purified to homogeneity. *B* - the rate of H_2O_2 accumulation measured in white (*open columns*) and pigmented (*closed columns*) sectors of corolla tissue. *C* - PRX activity measured in protein extracts from white and red sectors. Means from three separate measurements, *bars* represent SE.

Another important characteristic of the CHS mutant flower, is a sector specific rate of H_2O_2 accumulation and the total soluble PRX activity which resembles that for CHS protein. The rate of H_2O_2 accumulation in red sector represents 50 % of that measured in white sector. Similarly, the total soluble PRX activity was much lower in the red, CHS expressing, sector (decrease of activity at about 3-fold) than in the white sector (Fig. 1*B,C*).

H_2O_2 accumulation and PRX protein in different flower phenotypes: Since sector unspecific expression of PRX but, in turn, sector specific H_2O_2 accumulation was observed, we have established further whether these phenomena also correlated with increased expression of CHS protein. In six representative flowers (PH1 - PH6) which markedly differ in the percentual colour distribution (Fig. 2*A,B*) we investigated whether the level of pigmentation (CHS expression) correlates to the rate of H_2O_2 accumulation and total soluble PRX activity. While the highest rate of H_2O_2 accumulation was observed in completely white flowers (PH1), none H_2O_2 accumulation was detected in completely red flowers (PH6). The relatively low level of CHS expression (flower PH2) is sufficient to substantially reduce the rate of H_2O_2 accumulation (it represents almost 87 % when comparing the flowers PH1 and PH2, Fig. 3*A*). In contrary, total soluble PRX did not correlate with increasing amount of CHS expression even though the pattern of total PRX accumulation in flowers PH1 and PH6 resembled that observed for H_2O_2 accumulation.

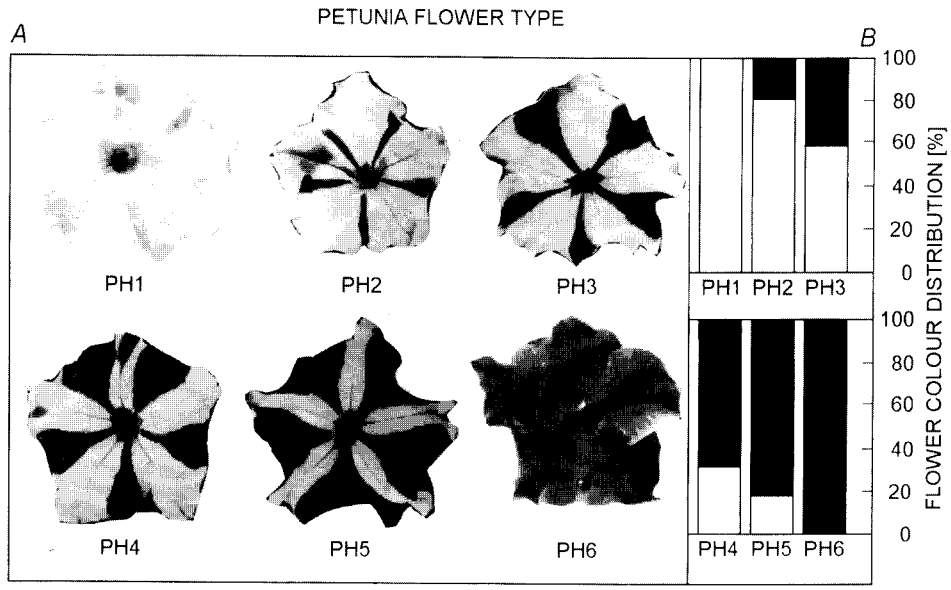

Fig. 2. Spatial distribution of pigmented and unpigmented sectors in six representative mutant flowers of petunia. *A* - flower phenotypes, *B* - computer-estimated flower colour distribution measured as % relative to completely red flower type (PH6).

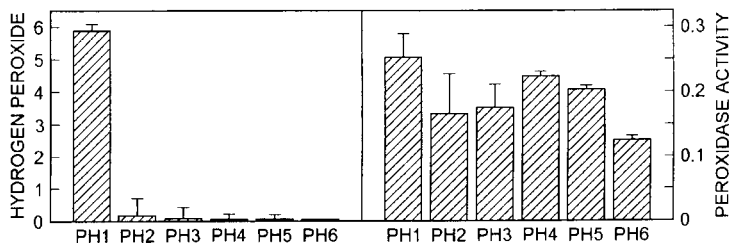

Fig. 3. Comparison of the H_2O_2 content (*A*) and total soluble PRX activity (*B*) in six representative mutant flowers. Means from three separate measurements, *bars* represent SE.

Western blot analysis using anti-srPRX antibody prepared against the purified cucumber srPRX was conducted with protein extracts from petunia flowers PH1 to PH6 and confirmed correlation between specific PRX and the rate of H_2O_2 accumulation (Fig. 4). Qualitative, as well as quantitative changes in the pattern of srPRX expression accompanied the decreasing H_2O_2 accumulation in respective flower types (Fig. 4). Qualitative changes in the pattern of srPRX expression reflect the isozyme spectrum comprising of three molecular forms designated as PRXa, PRXb and PRXc in this work. All three isoforms were detected in flowers

PH1 - PH3, while in flowers PH4 - PH6 the isoforms PRXa and PRXb disappeared completely. The highest rate of srPRX expression was detected in flower PH1 where it represents the value more than 2-fold greater than that in flower PH2.

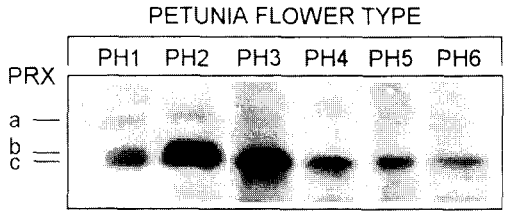

Fig. 4. Western blot analysis of 25 μg of total protein obtained from corollas of six representative mutant flowers. The blot was probed with anti-cucumber srPRX antibody and developed using chemiluminescence detection system. The lane PH1 contained 2-fold lower amount of protein loaded than lanes PH2 - PH6 to be comparable the level of signal intensity.

Effect of ABA and anther extirpation on PRX accumulation: When intact flower buds (stage 3) are grown on medium with sucrose, the corollas mature normally and become pigmented. However, when the anthers are extirped, the corolla stops growing and does not accumulate pigments due to block in CHS expression. Similarly, when the medium contains 10^{-5} M ABA, elongation and pigmentation is severely inhibited (Fig. 5*B*).

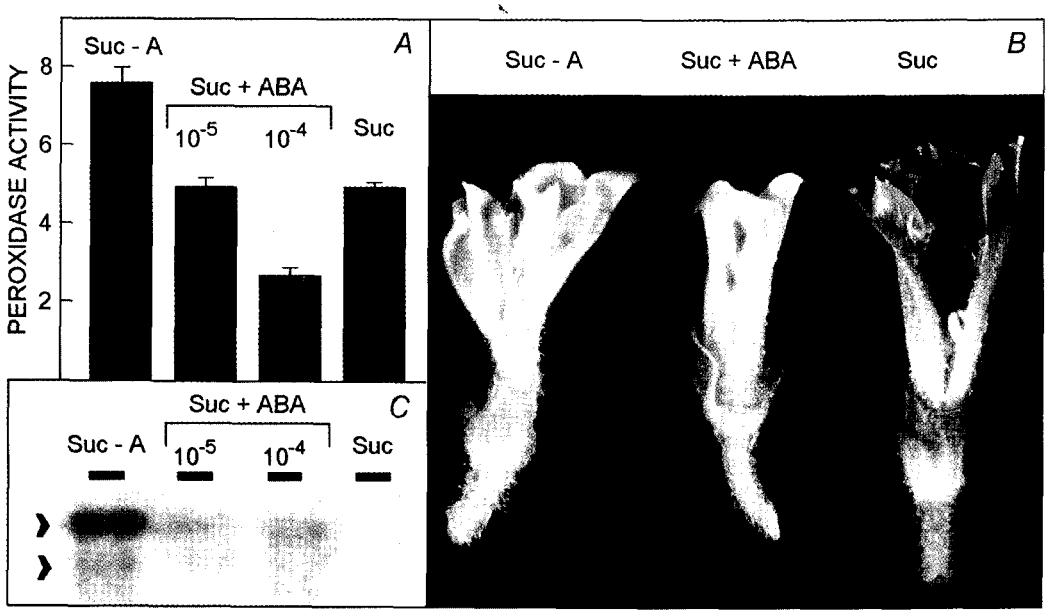

Fig. 5. The effects of sucrose (Suc), ABA (Suc + ABA) and anther extirpation (Suc - A) on total soluble PRX activity (*A*), corolla pigmentation (*B*), and accumulation of immunoprecipitable PRX in an *in vitro* cultured flower buds (*C*). Means from three separate measurements, *bars* represent SE. Protein was extracted 48 h after the onset of treatment and corresponding PRX was immunoprecipitated using specific anti-cucumber srPRX antibody. Immunoprecipitation mixture equal to 50 μg of total protein was loaded per lane of the 12.5 % SDS-PAGE gel and immunoprecipitated protein was detected using anti-srPRX antibody and chemiluminescence detection system. *Arrowheads* denote the prominent immunoprecipitated signals.

In vitro culture system and sucrose alone does not influenced either total PRX accumulation or immunoprecipitable srPRX accumulation. On the other hand, anther extirpation dramatically influenced the total soluble PRX accumulation under these semi *in vivo* growth conditions, as well as strongly increased immunoprecipitable signal on the Western blot (Fig. 5*A*). Interestingly, the immunopositive signal comprises of two isoforms with different rates of accumulation. ABA at concetration of 10^{-5} M did not inhibit accumulation of total soluble PRX when compared with sucrose, but unlike to this induces an accumulation of immuno-precipitable signal (Fig. 5*A*,*C*). ABA in lower concentration 10^{-8} to 10^{-6} M had no visible effects. Although the ABA at concentration of 10^{-4} reduced the rate of total PRX accumulation at about 50 % when compared with 10^{-5} M, the accumulation of immuno-precipitable signal seems to be concentration independent.

Discussion

In the present study we investigated the potential relationship between increased levels of H_2O_2 accumulation and srPRX activity. We have previously shown that srPRX strongly correlated with the development of hypersesitive response (HR) in cucumber leaves inoculated with an avirulent pathogen (Repka and Slováková 1994). Emerging data indicate that accumulation of H_2O_2 from the oxidative burst is a characteristic early feature of this HR following perception of pathogen avirulent signals (for review see Lamb and Dixon 1997). To estimate whether this srPRX can be involved in the direct detoxification of H_2O_2 excess or can substitute for the absence of flavonoid antioxidants we have used a model system based on the flowers of naturally occuring flavonoid-deficient mutant of *P. hybrida* (Red Star). The model benefits from both the serological cross-reactivity between petunia and cucumber PRX, and petunia mutant flowers differentially expressing CHS, a key enzyme of flavonoid biosynthesis. We found that in these mutant flowers the rate of CHS expression reflects the rate of H_2O_2 accumulation. Thus, the highest rates of H_2O_2 accumulation were characteristic for completely white flowers whose did not expresse the CHS protein. Moreover, we have also found that accumulation of H_2O_2 was accompanied with rise in anionic PRX expression. We speculate that the absence of these antioxidants in CHS mutant flowers may enhance both H_2O_2 accumulation and specific PRX. Such a hypothesis was supported by three independent findings. Firstly, relatively low level of CHS expression and thus flavonoids accumulation was sufficient to a marked decrease of the rate of H_2O_2 accumulation (flower PH2), even though the srPRX expression was still high as demonstrated on Western blot. This fact could be explained so that relatively small amounts of flavonoid antioxidants could scavenge the ROS serving as a source for H_2O_2 generation. Then, the remaining H_2O_2 is metabolized by enzymic antioxidants including PRX.

Secondly, Rao *et al.* (1996) demonstrated that UV-B and/or O_3 treated leaves of *Arabidopsis thaliana* flavonoid-deficient mutant *transparent testa* (*tt5*, a chalcon isomerase mutant) preferentially enhanced the accumulation of guaiacol-PRX, ascorbate PRX, and peroxidases specific to coniferyl alcohol. Taken together, in both mutants (CHS in petunia and CHI in *A. thaliana*) the inability to synthesize flavonoids would have generate substantially higher ROS and thus necessitates more efficient antioxidant defense mechanisms.

Thirdly, we showed previously that in various organs of cucumber male and female flowers the srPRX was strongly accumulated with the exception of both male and female petals, as well as the stamens (Repka and Jung 1995). Western blot analysis of the protein extracts from these flower organs revealed that neither in petals nor in anthers was srPRX accumulated. Interestingly, both these flower limb organs were rich in phenylpropanoid-derived compounds. Key among these were chalcones which were responsible for the yellow colour of anthers (De Vlaming and Kho 1976, Forkmann and Danglmayer 1980) whereas aurones contributed to the bright yellow colour of petals (Bohm 1988).

There are some difficulties, however, in interpretation of the above discussed findings. First is that flavonoids predominantly scavenge the hydroxyl radicals and/or singlet oxygen. Second, antioxidant enzymes including PRX act on less aggresive ROS (superoxide and H_2O_2) although the recent data indicate the possibility that many flavonoids can also act as scavengers of superoxide (Larson 1988, Yamasaki *et al.* 1996). We propose three possible mechanisms to explain the observed relationship between ROS production in absence of flavonoid antioxidants and elevated expression of specific PRX. In this context it is important to note that all models relate to ability of the interconversion of respective types of ROS.

The first model suggest that a small amounts of metabolically produced H_2O_2 could, in the presence of reduced transition metals such as Fe^{2+}, produce the very reactive hydroxyl radicals via Fenton reaction. Likewise other transition metal ions in a reduced form (especially Cu^{2+}) can similarly react with trace amounts of H_2O_2 and generate hydroxyl radical (Wardman and Candeias 1996). If not properly metabolized, *e.g.* in absence of flavonoid scavengers, hydroxyl radicals could react, besides with various organic molecules, each other to produce detrimental amounts of H_2O_2 which itself, in turn, can stimulate enhanced expression of enzymic antioxidant defense system including PRX. Although such a pathway has not yet been reported in plants, to be more exact in verification of this proposed model we simulated the elevated levels of ROS (especially H_2O_2) in the *in vitro* culture system. We have shown that inhibition of flavonoid synthesis either by exogenous ABA or pistil extirpation markedly stimulated the production of immunoprecipitable PRX. In contrast, if CHS expression, and thus flavonoid synthesis was not blocked, neither H_2O_2 nor immunoprecipitable PRX was observed.

The second model is a simpler interpretation of the recently reported data that PRX itself can also catalyze the formation of both superoxide and H_2O_2 by a complex reaction in which NADH is oxidized in plasma membrane (Morel *et al.* 1991, Babior 1992, Levine *et al.* 1994, Murphy and Auh 1996). If not properly metabolized by SOD and PRX, the superoxide anion and H_2O_2 can cross-react, and in presence of transition metals can undergo the Fenton reaction that gives rise to the extremely destructive hydroxyl radicals. From this point the second

model is almost identical to the first one. However, this model implies that either superoxide or H_2O_2 produced in many incompatible interactions (for review see Hammond-Kosack and Jones 1996) is not sufficiently metabolized by cellular enzymic antioxidant system. Such a speculation strongly supports the recent findings of Rao *et al.* (1996) that under specific situation (*e.g.* UV-B exposure of *A. thaliana* leaves) enhanced PRX preferentially over other antioxidant enzymes like SOD or CAT. It is important to take into account that UV-B induces the production of both the superoxide and the H_2O_2.

The simplest one is the model 3. It implies that metabolically produced superoxide is not safely metabolized by non-enzymic low-molecular antioxidants like flavonoids and thus is vulnerable to enzymatic (SOD) dismutation to H_2O_2. Accumulation of this ROS then could enhance the expression of PRX. The activity of flavonoid antioxidants seems to be a reaction of first order, since there are reports that at least in *Nicotiana*, SODs did not form part of the initial antioxidant response to O_3, SO_2, and UV-B. Northern blot analysis also confirmed that induction of some types of SOD occurs only with the onset of visible foliar damage (Willekens *et al.* 1994, Slooten *et al.* 1995). Furthermore, interesting finding that supports the former idea has recently been reported from research conducted on plants overexpressing the transcription factor AmMYB308 from *Antirrhinum*. Tamagnone *et al.* (1998a) have demonstrated that this factor, structurally related to the *c-MYB* protooncogene family of mammals (Sablowski *et al.* 1994), regulate different branches of flavonoid biosynthesis. Overexpression of the factor in transgenic tobacco plants represses phenolic acid metabolism resulting in precocious cell death (Tamagnone *et al.* 1998b). Since phenolic acids are known to be the most excellent antioxidants, rapid cell death may be viewed as a consequence of their absence in cells whose, thus not adequately protected against the toxic ROS, die rapidly.

Currently, however, more experiments are needed to further discriminate between the case of the models 1, 2 and 3, and potentially others not mentioned here.

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