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A stress-associated citrus protein is a distinct plant phospholipid hydroperoxide glutathione peroxidase

Talia Beeor-Tzahar, Gozal Ben-Hayyim, Doron Holland, Zehava Faltin, Yuval Eshdat*

Department of Fruit-Tree Breeding and Genetics, The Agricultural Research Organization, The Volcani Center, 50250 Bet Dagan, Israel

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Abstract **A protein whose level is markedly increased upon exposure of cultured citrus cells and whole plants to NaCl, was shown to specifically catalyze the reduction of phosphatidylcholine hydroperoxide in the presence of glutathione. This enzymatic activity was shown to be independent of a similar activity exhibited by glutathione *S*-transferase in plants. This finding corroborates the significant homology (52%) accounted between the deduced amino acid sequence of the gene encoding for this protein and that of mammalian phospholipid hydroperoxide glutathione peroxidases. While the mammalian enzyme is known and well investigated, this study establishes the presence of this key protein also in plants.**

Key words: Phospholipid hydroperoxide glutathione peroxidase; Stress; Citrus; Salt tolerance; Glutathione *S*-transferase

1. Introduction

Glutathione peroxidases (GPx)s are a family of multiple isozymes which catalyze the reduction of hydrogen peroxide, organic hydroperoxides and lipid peroxides by reduced glutathione, and help to protect the cells against oxidative damage [1,2]. Four distinct groups of these isozymes, characterized by comprising the rare Se-cysteine residue in their active site [3,4] were so far characterized in mammalians. These groups of isozymes differ in their structure, substrate specificity and tissue distribution [5]: the classical cellular and cytoplasmic glutathione peroxidase (c-GPx) [6], the plasma glutathione peroxidase [7,8], the cytosolic gastrointestinal glutathione peroxidase [5], and the phospholipid hydroperoxide glutathione peroxidase (PHGPx) [9–12]. The presence of GPx-activity in blue green cyanobacteria [13], chlamydomonas [14], marine diatoms [15], *Euglena* [16], moss [17], and leaves of several higher plants [18], and the isolation of c-GPx from *Aloe vera* [19], indicate that this enzyme might be widely spread among photosynthetic organisms. In some of these cases, the observed GPx-activity might be due to the presence of glutathione *S*-transferases (GST) in plants, known to exhibit GPx-activity in addition to their GSH-transferase catalytic action [20].

*Corresponding author. Fax: (972) (3) 9669583.

Abbreviations: GPx, glutathione peroxidase; GSH, reduced glutathione; c-GPx, cytoplasmic GPx; PHGPx, phospholipid hydroperoxide glutathione peroxidase; Cit-SAP, citrus salt-stress associated protein; GST, glutathione *S*-transferase; CSA$^-$, *E. coli* strain DH5α; CSA$^+$, *E. coli* strain DH5α transformed with *pARO1* plasmid which contains *csa*; CDNB, 1-chloro 2,4-dinitrobenzene.

Recently, two genes from tobacco and citrus [21,22], which were differentially expressed under stress conditions, were isolated and revealed significant sequence homology to those of mammalian GPx, the highest of which (52%) was towards PHGPx [10–12]. In both cases, the deduced amino acid sequence clearly suggests that the plant protein does not contain a selenocysteine residue in its presumed active site, in contrast to the animal enzyme, but a cysteine residue instead. One of these genes, designated *csa*, was isolated in our laboratory from cultured citrus cells that were acclimated to high levels of salt [22]. The protein product of *csa*, a citrus salt-stress associated protein (Cit-SAP), was purified in its denatured form by two-dimensional gel electrophoresis [23], and its partial amino acid sequence revealed identity with that deduced from the nucleotide sequence of *csa* [22]. Cit-SAP was found to be a cytosolic protein present in all citrus plant organs tested, and its level is increased in cultured cells exposed to NaCl and in citrus plants upon irrigation with salt [23]. In line with its sequence homology to PHGPx, the recombinant protein was shown to provide increased tolerance to oxygen radicals in transformed *E. coli* cells expressing Cit-SAP [24]. However, PHGPx as a distinct enzyme, so well known and studied as a key enzyme in animal cells, has not been isolated or characterized yet in plants. In the present study we have partially purified Cit-SAP from cultured citrus cells, separated it from the plant GST, and established its ability to catalyze specifically the reduction of phosphatidylcholine hydroperoxide by GSH. Thus, we have unequivocally demonstrated the existence of a phospholipid hydroperoxide glutathione peroxidase in plants.

2. Experimental

2.1. *Plant material, transformed* Escherichia coli *and protein extraction*

Adapted salt-tolerant cell lines derived from Shamuti orange (*Citrus sinensis* L. Osbeck) [25] were grown for two weeks in liquid Murashige and Tucker medium [26]. The cells were collected, washed by filtration with H_2O and with 50 mM Tris-HCl buffer (pH 7.0) containing 100 mM NaCl and 1 mM EDTA (Buffer A), homogenized by mortar and pestle, and centrifuged at 12,000 × *g* for 15 min to remove all non-soluble material. The supernatant was kept at 4°C for further experiments unless otherwise mentioned.

E. coli strain DH5α transformed with *pARO1* plasmid which contains *csa* (CSA$^+$ cells), and the same strain which does not contain *csa* but transformed only with the bluescript SK$^-$ vector (CSA$^-$ cells), were obtained as described before [24]. The cells were grown at 37°C for 15 h, in LB medium containing ampicillin (50 μg/ml) and 0.5 mM isopropyl β-D-thiogalactopyranoside. The cells were centrifuged at 4°C at 2,000 × *g* for 10 min, and the pellet was washed three times with 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. The washed cells were suspended in the same buffer (1/10, v/v), broken by freezing and thawing the suspension in liquid nitrogen (×2), and centrifuged at 12,000 × *g* for 15 min to remove all non-soluble material.

Protein concentration was determined according to Bradford [27], by using Bio-Rad Protein Assay kit and bovine γ globulin as standard.

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2.2. Materials

Activated thiol-Sepharose 4B and molecular weight markers were purchased from Pharmacia. Glutathione reductase (type III), GSH, GPx (bovine erythrocytes), lipoxygenase (type IV), NADPH, phosphatidylcholine Type III-B (chloroform solution), sodium deoxycholate (ultra grade), *S*-hexylglutathione and *S*-hexylglutathione- Agarose were obtained from Sigma. Acrylamide, bisacrylamide and TEMED were products of Bio-Rad. Alkaline phosphatase conjugated to anti-rabbit IgG antibodies was from Bio-Makor. All other chemicals were purchased from commercial companies and were of the highest purity available.

Phosphatidylcholine hydroperoxide was prepared by oxygenation with lipoxygenase according to Maiorino et al. [9], with minor modifications (i.e. lipoxygenase was added in three aliquots to the reaction mixture with vigorous shaking) as suggested by Graff et al. [28].

2.3. Purification of Cit-SAP by affinity chromatography

A column (0.5 × 1.5 cm) of activated thiol-Sepharose 4B was washed with buffer A containing 10 mM dithiothreitol. The gel was then washed with 3 volumes of Buffer A, and 1 ml of the protein extract obtained from the citrus salt-tolerant cells (10.4 mg of total protein) was loaded onto the column. After 14 h at room temperature, the column was washed with buffer A until no absorption at 280 nm was detected, followed by elution with buffer A containing 5 mM GSH. Fractions (0.6 ml) were collected and kept at −20°C until further use. The column was regenerated by subsequent washing with buffer A containing 10 mM dithiothreitol and buffer A.

2.4. Separation of Cit-SAP from glutathione S-transferase

Affinity separation of the citrus glutathione-*S*-transferase was done essentially according to Mozer et al. [29]. A column (0.5 × 1 cm) of *S*-hexylglutathione-Agarose (Sigma) was prewashed with 10 mM Tris-HCl buffer (pH 8) containing 200 mM NaCl and 1 mM EDTA (Buffer B), and 1 ml of the protein extract obtained from the citrus salt-tolerant cells (4.5 mg of total protein) was loaded onto the column. After 14 h at room temperature, the column was washed with buffer B until no absorption at 280 nm was detected, followed by elution with the washing buffer containing 5 mM *S*-hexylglutathione and 2.5 mM GSH. Fractions (0.3 ml) were collected and kept at −20°C until further use.

2.5. Enzymatic assays

Phospholipid hydroperoxide GPx activity (PHGPx-activity) was measured at 23°C by a modification of the assay described by Maiorino et al. [9]. The assay mixture (1 ml total volume) contained 100 mM Tris-HCl buffer (pH 8.2), 5 mM EDTA, 15 mM GSH, 0.15 mM NADPH, 3 units of glutathione reductase and the examined protein fraction. After 5 min of preincubation, phosphatidylcholine hydroperoxide was added to a final concentration of 20 μM and the rate of the change in the absorbance at 340 nm was monitored. In calculating the rate of activity, the absorbance change in the absence of phosphatidylcholine hydroperoxide in the assay mixture was taken into account; no change in the absorbance was detected when either GSH or the protein sample was omitted from the reaction mixture.

The cGPx-activity (glutathione:hydrogen-peroxide oxido-reductase) was determined under different conditions as described [14,18,30]. Bovine erythrocytes GPx and chlamydomonas protein extract were used as positive control tests of this enzymatic reaction.

Determination of Glutathione *S*-transferase activity (GST-activity) was done according to Habig [31], using, as substrates, 1 mM 1-chloro 2,4-dinitrobenzene (CDNB) and 1 mM GSH in 100 mM Sodium-Phosphate buffer (pH 6.5).

Inhibition of the peroxidase activity of GST was done by preincubation of the protein extract (880 μl) in the presence of 23 mM *S*-hexylglutathione [32], prior to the addition of 120 μl of the substrates.

2.6. Gel electrophoresis

PAGE in the presence of SDS and immunoblotting of Cit-SAP were done as previously described [23]. Western blots were analyzed by reactions with anti-Cit-SAP antibodies and by staining with alkaline phosphatase conjugated to a second antibody. Determination of the molecular weight of native Cit-SAP was done by non-denaturing PAGE using the reagents kit and protocol supplied by Sigma, which is based on the method described by Bryan [33].

3. Results

3.1. Affinity purification of Cit-SAP

Affinity chromatography of the protein extract obtained from the citrus salt-tolerant cells on a Sepharose-GSH column is shown in Fig. 1. Immunoblotting of aliquots taken from each fraction demonstrates that Cit-SAP was eluted in both the washing peak (A1, unbound) and the peak eluted in the presence of GSH (A2, bound). Fractions containing Cit-SAP in A1 and A2, separately, were pooled together for quantitation of the relative amount of Cit-SAP (by immunoblotting) and of the level of GPx- and GST-activities in the two peaks. Approximately 60% of Cit-SAP is present in peak A1, and 40% in peak A2 (Fig. 2, lanes 7, 8). The PAGE-SDS protein profile of samples taken from the two peaks (Fig. 2, lanes 2, 3) demonstrates that the bound Cit-SAP, which was specifically eluted by GSH, was significantly enriched. Enzymatic assay for PHGPx-activity and GST-activity revealed (Table 1) that PHGPx-activity was present in both protein peaks while GST-activity was measured only in the washing peak A1.

3.2. Separation of Cit-SAP from glutathione S-transferase

Affinity chromatography of the crude protein extract obtained from the citrus salt-tolerant cells on a *S*-hexylglutathione-Agarose column is shown in Fig. 3. Immunoblotting with anti-Cit-SAP antibodies revealed that Cit-SAP was completely washed from the column with the unbound material (peak B1). On the other hand, the enzymatic assay of GST-

Table 1
PHGPx and GST enzymatic activities of protein extract obtained from salt-tolerant cultured citrus cells, which was separated by affinity chromatography on either GSH-Sepharose or *S*-hexylglutathione-agarose columns

Enzymatic activity (units)[a]	GSH-Sepharose column			*S*-hexyl-GSH-agarose column		
	Cell extract	Peak A1	Peak A2	Cell extract	Peak B1	Peak B2[b]
PHGPx						
Total activity	41.6	30.7	5.7	19.2	2.9	6.9
Specific activity	4.0	3.4	27	4.3	1.1	138
GST						
Total activity	2,374	1,874	0	916	12.5	681
Specific activity	228	206	0	203	4.8	13,620

[a] One unit of enzymatic activity is defined as the amount of enzyme which oxidizes 1 nmol NADPH/min (for PHGPx) or 1 nmol CDNB/min (for GST). The specific activity is presented in units/mg protein.

[b] Since 5 mM *S*-hexyl-glutathione, an inhibitor of GST, were included in the protein samples taken from peak B2, appropriate corrections (30% for PHGPx-activity and 67% for GST-activity) were made to obtain the final calculated values.

activity (Table 1) demonstrates that 98% of GST was specifically bound to the column and eluted only by the addition of *S*-hexylglutathione and GSH to the buffer.

3.3. Determination of the molecular weight of native Cit-SAP

Ferguson plots of standard proteins and of Cit-SAP, deduced from the relative mobility of these proteins in different gel concentrations, are shown in Fig. 4. According to the plot of the retardation coefficients of the standard proteins (calculated from the Ferguson plots) versus their known molecular weight, the molecular weight of the native Cit-SAP is approximately 25 kDa.

3.4. GPx and GST enzymatic activity of the protein fractions

The values obtained for the PHGPx-activity and the GST-activity of the citrus cells' protein fractions separated by affinity chromatography are presented in Table 1. PHGPx-activity (using phosphatidylcholine hydroperoxide as a substrate) was measured in all the fractions which contained either Cit-SAP or glutathione *S*-transferase. On the other hand, cGPx-activity (using hydrogen peroxide as substrate) was not detected in any of the protein samples examined under the various assay conditions used [14,18,30]. Addition of 2 mM GSH to the protein cell extract, known to protect against loss of cGPx-activity after cell disruption [19], did not result in any detectable cGPx-activity in the extract.

In order to obtain an additional estimation of the portion of PHGPx-activity which is due to the presence of GST in the citrus cell extract, inhibition study with *S*-hexylglutathione (known to inhibit specifically the enzyme GST [32]) was done. Indeed, 93% of the PHGPx-activity of the citrus GST isolated by affinity chromatography (see section 3.2. and Table 1) was inhibited by *S*-hexylglutathione. Determination of the PHGPx-activity of the protein extract obtained from the citrus cultured cells in the presence of this inhibitor has shown that *S*-hexylglutathione inhibits 67% of the PHGPx-activity. This result suggests a ratio of approximately 2:1 between the PHGPx-activity of GST to that of Cit-SAP in the citrus cell extract.

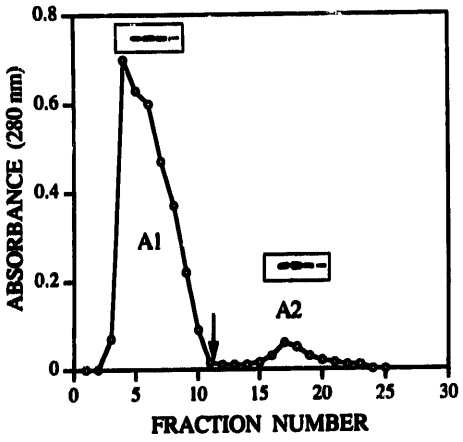

Fig. 1. Partial purification of Cit-SAP from cultured citrus cell extract by affinity chromatography on GSH-Sepharose column. Arrow presents the point of GSH addition to the washing buffer. Western blot analysis using anti-Cit-SAP antibodies, of aliquots (10 μl) taken from fractions 4–8 (peak A1) and of aliquots (20 μl) taken from fractions 16–20 (peak A2), are shown. Silver stain analysis of SDS-PAGE of aliquots taken from the two peaks are shown in Fig. 2. For experimental details see text.

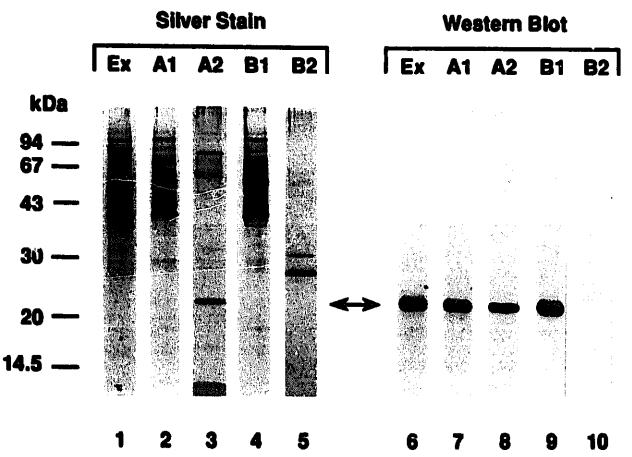

Fig. 2. SDS PAGE of protein fractions obtained after affinity chromatography of citrus cultured cell extract on GSH-Sepharose and *S*-hexylglutathione-Agarose columns (Fig. 1 and 3). Lanes 1–5: Silver stain analysis of the protein extract (1), fraction 6 of peak A1 (2), fraction 18 of peak A2 (3), fraction 6 of peak B1 (4), fraction 23 of peak B2 (5); equal amounts of total protein (0.25 μg) were loaded onto each well. Lanes 6–10: Western blot analysis of Cit-SAP in the protein extract (6), fractions 5–8 of peak A1 (7), fractions 17–20 of peak A2 (8), fractions 6–9 of peak B1 (9), fractions 22–25 of peak B2 (10); equal amounts (0.3% of each collected peak) were loaded onto each well. Arrows point to the position of Cit-SAP. For experimental details see text.

Protein extracts obtained from CSA^+ and CSA^- transformed *E. coli* were also examined for their GST and GPx activities. No GST activity was observed in both bacterial populations. However, while CSA^+ protein extract exhibited PHGPx specific activity of 31 units/mg protein, CSA^- was completely devoid of such enzymatic activity.

4. Discussion

In plants, oxidative stress causes the induction of the Halliwell-Ashada pathway enzymes (for review see [34,35]). Following dismutation of superoxide radicals into H_2O_2 and O_2 by superoxide dismutase, ascorbate peroxidase reduces H_2O_2 into water by converting ascorbate into dehydroascorbate. The later is reduced back to ascorbate by GSH, and the resulted GSSG would be reduced back to GSH by glutathione reductase. In animals, GPx has been demonstrated to play the key role as a major scavenger of H_2O_2. Indication for such GPx-activity in plants was reported some 10 years ago [18], but only recently a protein isolated from *Aloe vera* was shown to exhibit similar enzymatic activity and molecular properties to the selenoprotein c-GPx [19]. In our earlier study [36], a partial amino acid sequence analysis of a protein (Cit-SAP) isolated from salt-stressed citrus cultured cells revealed significant similarity to animal GPx. This finding was further supported by the nucleotide sequence determined for the cloned citrus gene (*csa*) expressing this protein [22]. A notable difference between the deduced amino acid sequence of the citrus protein and the animal GPx is the presence of an active-site cysteine residue in the plant protein as compared to a selenocysteine residue in GPx. A similar sequence data was obtained from a cDNA clone isolated from tobacco cells [21], but no characterization or purification of the tobacco protein product was so far reported.

Our present study was aimed to identify whether the primary structure resemblance of Cit-SAP to GPx also reflects a similar biological activity. Examination of a possible enzymatic activity similar to that of animal cGPx (namely, catalysis of the reduction of H_2O_2 by GSH), was extensively studied under various conditions. Although Cit-SAP exhibits 38% sequence homology to the mammalian cGPx [22], no indication for any cGPx-activity was observed for the protein fraction containing Cit-SAP isolated from citrus cultured cells. Moreover, our present results show that Cit-SAP is a monomer in its native state, while cGPx is a tetramer [4].

Recent studies [10–12] imply that the degree of homology between the deduced amino acid sequence of *csa* and that of the gene encoding for mammalian PHGPx is higher (52%) than that between Cit-SAP and cGPx. Moreover, the mammalian PHGPx is monomeric in its native form [30] as we have found for Cit-SAP. Indeed, PHGPx-activity was observed in the crude protein extract obtained from the adapted salt-tolerant citrus cells (Table 1). However, since plant glutathione *S*-transferases are known to exhibit PHGPx-activity in addition to their transferase activity, we devised methods to separate between Cit-SAP and the citrus GST by affinity chromatography. We have used two different resins, GSH-Sepharose and *S*-hexylglutathione-Agarose, which were shown to specifically bind either Cit-SAP or citrus GST, respectively (Fig. 1 and 3). As shown in Fig. 1 and Table 1, approximately 40% of Cit-SAP was bound to the GSH-Sepharose column and was specifically eluted with GSH (Fig. 1, peak A2), while all the GST did not bind to the column (Table 1, peaks A1 and A2). Although Cit-SAP was not completely purified by its affinity chromatography on this column (Fig. 2, peak A2), it was significantly enriched (by approximately 20-fold) as compared to the original protein extract (Table 1 and Fig. 2).

Chromatography on *S*-hexylglutathione-Agarose column provided a complete separation between Cit-SAP, that was washed from the column (Fig. 3, B1), and GST that was bound to the resin and was eluted by *S*-hexylglutathione and GSH (Fig. 3, B2, and Table 1). From the distribution of PHGPx-activity determined in peaks B1 and B2, it can be concluded

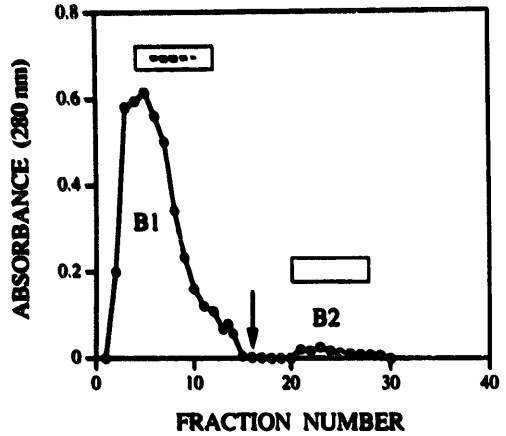

Fig. 3. Separation of Cit-SAP from citrus GST by affinity chromatography on *S*-hexylglutathione-Agarose column. Arrow presents addition of 5 mM *S*-hexylglutathione and 2.5 mM GSH to the washing buffer. Western blot analysis using anti-Cit-SAP antibodies of fractions 5–11 (peak B1) and fractions 21–27 (peak B2) are shown. Silver stain of SDS-PAGE of aliquots taken from the two peaks are shown in Fig. 2. For experimental details see text.

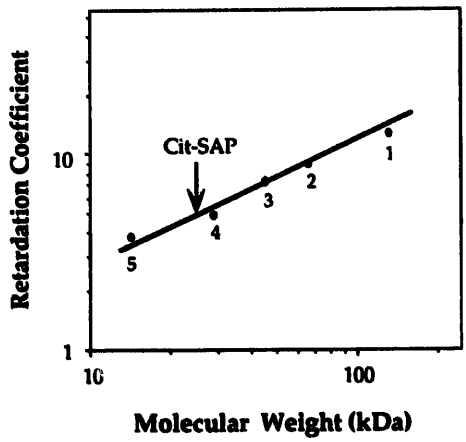

Fig. 4. Determination of the apparent molecular weight of non-denatured Cit-SAP according to Bryan [33]. Molecular markers are: (1) Bovine Serum Albumin (dimer); (2) Bovine Serum Albumin (monomer); (3) Chicken Egg Albumin; (4) Carbonic Anhydrase; (5) α-Lactalbumin. Arrow points to the intersection between the retardation coefficient value obtained for Cit-SAP and the calibration curve calculated form the retardation coefficient values obtained for the markers and their known molecular weights.

that approximately 70% of the PHGPx-activity in the protein extract is due to GST, and 30% is due to a protein located in peak B1. Further support to this distribution of PHGPx-activity was obtained by measuring the PHGPx-activity in the presence of *S*-hexylglutathione, shown to inhibit GST. When the enzymatic activity of GST was specifically inhibited by the presence of *S*-hexylglutathione in the citrus protein extract, only 33% of the original PHGPx-activity was detected. It can be therefore concluded that a protein other than GST is responsible for approximately 30% of the PHGPx-activity in the citrus protein extract.

The results of the affinity separation on GSH-Sepharose column (Fig. 1 and Table 1) suggest that the citrus protein carrying the PHGPx-activity, in addition to GST, is indeed Cit-SAP. Although only about 40% of Cit-SAP was bound to the column and eluted with GSH (Fig. 1 and 2, peak A2), the elevated enrichment of Cit-SAP in the elution peak (which is completely devoid of GST) corroborates with the increase of the PHGPx specific activity observed for this peak (Table 1). The finding that the protein extract of *E. coli* cells containing the recombinant Cit-SAP exhibits PHGPx activity, strongly support the above conclusion that Cit-SAP is the plant analog of the animal PHGPx. Therefore, the results obtained in this study indicate that the amino acid sequence homology found to exist between Cit-SAP and the mammalian PHGPx reflects similarity in the specific biochemical activity as well, and demonstrate that Cit-SAP is indeed a plant phospholipid hydroperoxide glutathione peroxidase.

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