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An Inducible Glutathione *S*-Transferase in Soybean Hypocotyl Is Localized in the Apoplast

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Glutathione *S*-transferases (GSTs) with additional activities as fatty acid hydroperoxidases were investigated in soybean (*Glycine max* L.) hypocotyls. Aside from the GSTs present in total soluble tissue extracts, enzyme activities and distinct immunoreactive GST polypeptides were also detected in the intercellular washing fluid. Whereas the intracellular isoenzymes were both constitutive and inducible, apoplastic GST and glutathione peroxidase was detectable only in tissues treated with the known GST inducer 2,3,5-triiodobenzoic acid. Monensin inhibited the induced accumulation of apoplastic GST but did not affect the intracellular isoforms. The discovery of apoplastic inducible GST will be discussed in light of the putative function of these enzymes in plants.

The GSTs (EC 2.5.1.18) are a family of proteins with several activities (Wilce and Parker, 1994; Marrs, 1996). GSTs catalyze the nucleophilic attack of the thiol of GSH to electrophilic substrates, typically resulting in the formation of GSH conjugates. GSTs are usually dimeric proteins with subunit molecular masses of 24 to 30 kD; they are organized in gene families that produce multiple isoenzymes in eukaryotic organisms. GSTs are mostly soluble cytoplasmic enzymes, but microsomal isoforms are also known in both plants and animals. GSTs conjugate GSH to various xenobiotics, e.g. drugs and pesticides, which is often a key step in their metabolic detoxification and elimination from the cytoplasm (Lamoureux and Rusness, 1989; Kreuz et al., 1996; Reinemer et al., 1996).

The multitude of GST isoenzymes in plants and their inducibility by diverse biotic and abiotic factors has been the primary focus of research in recent years. Yet, knowledge about the physiological substrate(s) and function(s) of plant GSTs is only slowly beginning to emerge. Thus, GSTs have been implicated in the conjugation of cinnamic acid and of anthocyanins and in the binding of IAA (Edwards and Dixon, 1991; Marrs, 1996). Some GST isoenzymes display additional activities; for example, the selenium-independent GSH peroxidases catalyze the reduction of fatty acid hydroperoxides with concomitant formation of GSSG (Bartling et al., 1993). Such hydroperoxides are formed by the action of active oxygen species that are generated both as normal by-products of aerobic metabolism and as the result of pathogen infection or exposure to certain abiotic agents. Organic hydroperoxides are potentially cytotoxic, and their removal by GSH peroxidase activity has thus been implicated in the protection of tissues against oxidative stress (Ketterer et al., 1990).

In this report we describe the isolation and characterization of GSTs from soybean (*Glycine max* L.) that display high GSH peroxidase activity toward hydroperoxides of linolenic acid and arachidonic acid. In addition to the soluble intracellular isoenzymes, GST and GSH peroxidase were also found in the intercellular washing fluid of hypocotyls. These apoplastic enzyme activities and immunoreactive apoplastic GST polypeptides were only detectable after treatment of tissues with TIBA, a known inducer of soybean GST. To our knowledge, this is the first report of extracellularly localized GST in plants.

MATERIALS AND METHODS

Soybean (*Glycine max* L. Merr. cv Asgrow) seeds were planted in moist vermiculite and grown for 7 d at 27°C and 80% RH, with a 16-h photoperiod and a PPFD of 100 μmol m^{-2} s^{-1}. For GST induction the plant roots were excised under water and the cut ends were placed into tap water containing 100 μM TIBA and 0.1% (v/v) DMSO. The plants were returned to the growth chamber for 24 h of continuous illumination prior to enzyme extraction.

Purification of GST

All operations were conducted at 4°C. Enzyme purification was performed as previously described (Flury et al., 1995) with minor modifications. In brief, hypocotyl tissue was pulverized under liquid nitrogen with a mortar and pestle. The powder was suspended in 3 volumes of 100 mM sodium phosphate (pH 7.0), 5 mM DTT, 1 mM EDTA, and 0.1 mM PMSF. The suspension was filtered through cheesecloth and centrifuged for 15 min at 15,000*g*. Protein in the supernatant was precipitated at 80% $(NH_4)_2SO_4$ saturation and resuspended in buffer A (10 mM sodium phosphate, pH 7.3). Following dialysis overnight against buffer A, the protein was loaded onto a column of DEAE-Sepharose FF (Pharmacia). Bound protein was eluted with a linear gradient of 0 to 0.4 M NaCl in buffer A, and fractions with GST

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; FPLC, fast protein liquid chromatography; GST, glutathione *S*-transferase; TIBA, 2,3,5-triiodobenzoic acid.

activity toward CDNB were pooled. Protein in the pooled fractions was precipitated at 80% $(NH_4)_2SO_4$ saturation, resuspended in buffer A, and desalted on Sephadex G-25 columns (PD-10, Pharmacia). The desalted protein was subjected to affinity chromatography on *S*-hexylglutathione agarose (Sigma). Bound GST was eluted with 50 mM GSH in 10 mM sodium phosphate (pH 8.3), and fractions with GST activity were pooled.

FPLC Chromatography of GST

Anion-exchange FPLC was performed essentially according to the method of Fuerst et al. (1993). The GST purified by affinity chromatography (see above) was loaded (about 3 mg of total protein) onto a Mono-Q HR 5/5 column (Pharmacia). The column was washed with buffer A until the baseline was reached, and bound GST was eluted by applying a 100-mL linear gradient of 0 to 250 mM NaCl in buffer A at a flow rate of 1 mL min^{-1}. Protein elution was monitored by A_{280}, and fractions of 1 mL were collected and assayed for GST activity.

Preparation of Apoplastic, Microsomal, and Total Soluble Protein

Apoplastic protein was extracted from 1.5-cm-long hypocotyl segments by gentle vacuum infiltration with 20 mM potassium phosphate (pH 7.0), followed by centrifugation at 1000*g* for 10 min to recover the infiltrate (Van Loon and Gerritsen, 1989).

For the preparation of microsomal and total soluble protein, hypocotyls were minced with a scalpel and homogenized in 3 volumes of homogenization buffer containing 100 mM Tris-HCl (pH 7.5), 500 mM Suc, 10 mM $MgCl_2$, 5 mM DTT, and 1 mM EDTA, using a homogenizer (Polytron, Kinematica, Littau, Switzerland) twice for 1 min. The homogenate was passed through two layers of Miracloth (Calbiochem) and centrifuged at 6000*g* for 10 min; then the supernatant was centrifuged at 105,000*g* for 90 min. The resulting supernatant, designated the "total soluble fraction," was used for the subsequent immunoblotting and enzyme assays. The microsomal pellet was washed by resuspending it in homogenization buffer and recentrifuging. All operations were performed at 4°C.

Enzyme Assays

GST activity with CDNB as substrate was assayed as described by Fuerst et al. (1993), except that the pH of the assay buffer was adjusted to pH 6.8. The results obtained by this method were initially confirmed by an HPLC-based enzyme assay (Kreuz et al., 1989). GSH peroxidase activity was measured according to the method of Simmons et al. (1989). The hydroperoxides of linolenic acid and arachidonic acid used as GSH peroxidase substrates were prepared by the method of Graff et al. (1990). This method has been reported to yield mainly 13-hydroperoxy-9, 11,15-octadecatrienoic acid and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid, respectively. Glc-6-P dehydrogenase was assayed spectrophotometrically. Protein was measured, after precipitation with 7% (w/v) TCA, by the procedure of Lowry et al. (1951) in the presence of 1% (w/v) SDS.

Electrophoresis and Immunoblotting

For one-dimensional SDS-PAGE, tissue samples were extracted with electrophoresis sample buffer and subjected to SDS-PAGE in 12% acrylamide gels as previously described (Flury et al., 1995), using the buffer system of Laemmli (1970). For two-dimensional PAGE, protein was extracted by the method of Barent and Elthon (1992) and subjected to first-dimension IEF according to the procedure of O'Farrell (1975) but using 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate as the detergent and piperazine diacrylamide instead of bis-acrylamide. Second-dimension SDS-PAGE was performed as previously described using 12% acrylamide gels.

Following electrophoresis, proteins were electroblotted onto nitrocellulose membranes. The blots were probed with a rabbit antiserum raised against a mixture of 26- and 27-kD GST subunits from maize (Flury et al., 1995) and developed by using an enhanced chemiluminescence-based detection system (ECL, Amersham).

RESULTS

Purification and Characterization of GST

Plants used for GST purification were pretreated with 100 μM TIBA for 24 h, which increased both the GST activity and the level of immunoreactive GST protein by a factor of 7 to 8, as compared with untreated plants (Flury et al., 1995). GST was isolated from soybean hypocotyls using affinity chromatography on *S*-hexylglutathione agarose as the major purification step (Flury et al., 1995). The affinity-purified GST fraction was subsequently further resolved by anion-exchange FPLC on a Mono-Q column (Fig. 1A). Both the protein profile and the GST activity toward CDNB as substrate displayed five partially resolved peaks. Two-dimensional PAGE of these peak fractions revealed the presence of two groups of polypeptides with apparent molecular masses of 26 and 28 kD, respectively, and with pI values of individual polypeptides between 6.4 and 5.8 (data not shown). These polypeptides cross-reacted with an antiserum raised against GST from maize (Fig. 2), which supports the conclusion that they represent subunits of several GST isoforms. Fuerst et al. (1993) also detected multiple GST polypeptides in maize in the 27- to 29-kD range by means of two-dimensional PAGE coupled to immunoblotting.

The enzymatic properties of three selected peak fractions (Fig. 1A, arrows) were analyzed further. They displayed GST activities toward CDNB that were enriched 505-fold (fraction 57), 520-fold (fraction 67), and 567-fold (fraction 80) as compared with the GST activity in the crude extract of TIBA-treated hypocotyls. These GST fractions had distinct K_m values for CDNB of 434 ± 103, 560 ± 53, and 319 ± 37 μM, respectively. Furthermore, these GST fractions showed appreciable activities as GSH peroxidases toward hydroperoxides of linolenic acid and arachidonic acid (Fig. 1B). The GSH peroxidase activities were not inhibited by

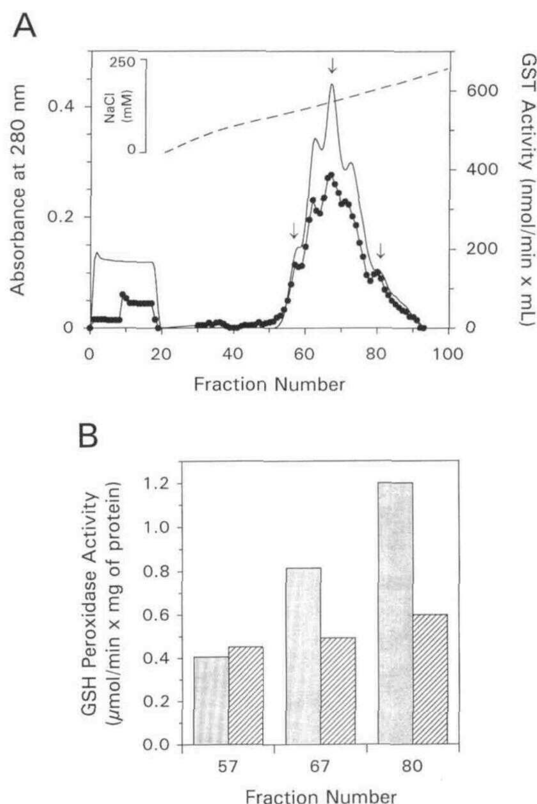

Figure 1. *Anion-exchange chromatography of GST on Mono-Q (A) and GSH peroxidase activity (B).* A, GST activity toward CDNB as substrate (●). Vertical arrows indicate fractions that were analyzed in B. B, GSH peroxidase activity toward hydroperoxides of linolenic acid (shaded bars) and arachidonic acid (hatched bars).

sodium azide, which clearly distinguishes these enzymes from the selenium-dependent GSH peroxidases.

GST in the Apoplastic Fraction

To investigate the subcellular distribution of GST, the crude extract of TIBA-induced hypocotyls was fractionated into a soluble fraction and microsomal membranes (see "Materials and Methods"). In addition, an intercellular washing fluid, designated the "apoplastic fraction," was prepared from hypocotyls according to established procedures (Van Loon and Gerritsen, 1989). These fractions were assayed for GST and GSH peroxidase activity and for immunoreactive GST polypeptides using the antiserum to maize GST.

The highest specific activity of GST was found in the soluble fraction (Table I). Microsomal membranes contained variable but generally very low GST activities, which could be readily washed off by resuspension/recentrifugation (data not shown). However, considerable GST activity was detected in the apoplastic fraction. The cytosolic marker enzyme Glc-6-P dehydrogenase was present in very low to undetectable activities in the intercellular washing fluid, indicating virtually negligible contamination of the apoplastic fraction with cytosolic protein (Table I). Immunoblot analysis following two-dimensional PAGE

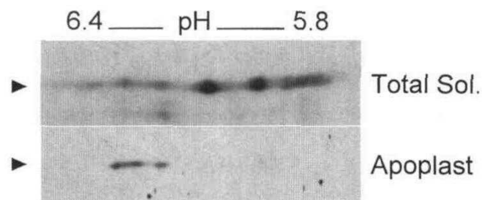

Figure 2. Immunoblot analysis of total soluble and apoplastic GST. Proteins in the total soluble fraction (Total Sol.) and in the apoplastic fraction (Apoplast) were separated by two-dimensional PAGE prior to immunoblotting. Arrowheads indicate the estimated molecular mass of 26 kD.

of the proteins in the soluble fraction and in the apoplastic fraction indicated the presence of GST polypeptides in accordance with the relative levels of GST enzyme activities (Fig. 2). The apoplastic fraction contained predominantly the two less-acidic polypeptides of 26 kD, which were also found in the total soluble extract. No GST polypeptides could be detected on immunoblots of apoplastic proteins from noninduced hypocotyls (data not shown).

Whereas GST and GSH peroxidase activities were present in the total soluble hypocotyl extract from both untreated and TIBA-treated plants, no constitutive enzyme activities could be detected in the apoplastic fraction (Table II). Considerable GST and GSH peroxidase activities, however, were found in the apoplastic fraction of hypocotyls previously induced with TIBA.

Effect of Monensin on GST

The presence of both GST activity and distinct polypeptides immunologically related to GST suggested the specific deposition of GST isoforms in the apoplastic space of the hypocotyl tissue in response to induction with TIBA. To corroborate the export of GST into the extracellular space, experiments were conducted with the known inhibitor of the protein secretory pathway, monensin (Mollenhauer et al., 1990). Monensin strongly decreased the GST activity in the intercellular washing fluid of TIBA-treated hypocotyls, whereas the GST activity in the total soluble fraction remained virtually unchanged with up to 5 μM monensin (Fig. 3A). At 5 μM monensin, the apoplastic GST activity was 24 ± 6% of the activity in untreated hypocotyls, whereas the total soluble GST activity still accounted for 86 ± 11% of the control. The results obtained by immunoblot analysis following SDS-PAGE were in excellent agreement with these findings (Fig. 3B). GST protein accumulation in the intercellular washing fluid was progressively

Table I. *Activities of GST and Glc-6-P dehydrogenase*

Hypocotyls were treated with 100 μM TIBA for 24 h prior to the preparation of the apoplastic and total soluble fractions. Values are means ± SD (n = 3).

Fraction	GST	Glc-6-P Dehydrogenase
	nmol min^{-1} mg^{-1} protein	
Total soluble	152 ± 29 (100%)	307 ± 36 (100%)
Apoplastic	70 ± 13 (46%)	0 – 15 (<5%)

Table II. *Induction of GST and GHS peroxidase activities*

Hypocotyls were treated in the absence or presence of 100 μM TIBA for 24 h prior to the preparation of the apoplastic and total soluble fractions. GSH peroxidase activity was assayed with linolenic acid hydroperoxide as substrate. Values are means ± SD (n = 3).

Fraction	GST		GSH Peroxidase	
	Control	TIBA	Control	TIBA
	nmol min⁻¹ mg⁻¹ protein			
Total soluble	24 ± 5	152 ± 29	28 ± 7	43 ± 3
Apoplastic	n.d.[a]	70 ± 13	n.d.	25 ± 3

[a] n.d., Not detectable.

inhibited by increasing the monensin concentrations, whereas the total soluble GST protein concentration remained virtually unchanged.

DISCUSSION

The present work provides evidence for the existence of inducible GST in the apoplastic space of soybean hypocotyls. Evidence is based on the detection of GST activity in the intercellular washing fluid, which was virtually devoid of cytoplasmic contamination. Furthermore, of the several 26-kD polypeptides recognized by a GST antiserum among the total soluble hypocotyl proteins, two polypeptides with distinct pIs were detected in the apoplastic fraction. Finally, monensin, a drug known to perturb the Golgi-based secretory pathway (Mollenhauer et al., 1990), inhibited the accumulation of both enzyme activity and immunoreactive polypeptides in the extracellular fraction of induced hypocotyls. Extracellular GST was detectable only in hypocotyl tissue that had been treated with TIBA, a compound that had previously been shown to be a potent inducer of GST in soybean (Flury et al., 1995).

The present contribution provides, to the best of our knowledge, the first indication of extracellularly localized GST in plants. Extracellular GST has so far been described only in certain parasitic nematodes (Liebau et al., 1994; Brophy et al., 1995). It is thought that these GSTs are part of the worm's defense system against products of lipid peroxidation caused by immune attack of the host. One particular nematode GST contains an N-terminal extension that could function as a signal sequence for secretion. In recent years, cDNA and gene sequences of a number of plant GSTs have been analyzed (Droog et al., 1993; Jepson et al., 1994). However, none of these studies revealed the presence of potential signal sequences in GSTs that would enable their transport across the plasma membrane.

Various isoforms of soybean GST purified to apparent homogeneity displayed high GSH peroxidase activity toward unsaturated fatty acid hydroperoxides. To date, GSTs with additional activities as fatty acid hydroperoxidases have been demonstrated in few plant species, including pea, wheat, and Arabidopsis (Williamson and Beverley, 1987, 1988; Bartling et al., 1993). Furthermore, we suggest that the inducible GSH peroxidase activity found in the apoplast is actually catalyzed by GST. The insensitivity of the apoplastic GSH peroxidase toward sodium azide and the absence of activity with H_2O_2 as substrate clearly distinguish this enzyme from the seleno-GSH peroxidases (Ketterer et al., 1990). In plants conclusive evidence for both enzyme activities residing on one GST protein has until now been provided only by the heterologous expression of a GST cDNA from Arabidopsis (Bartling et al., 1993).

In some leguminous species such as soybean, homoglutathione (γ-glutamylcysteinyl-β-Ala) functionally replaces GSH in vivo (Rennenberg, 1982). Homoglutathione has been detected in the intercellular washing fluid of soybean hypocotyls (data not shown) in accordance with previous reports (Rennenberg, 1982; Jamaï et al., 1996). Recent studies showed that the plasma membrane of broad bean leaf cells contains a specific transport system that imports GSSG and GSH conjugates preferentially over GSH (Jamaï et al., 1996). It may thus be inferred that extracellular GSH conjugates of xenobiotics, for example, possibly produced by the apoplastic GST, are transported into the cell for detoxification as proposed by Jamaï et al. (1996). GSSG generated by extracellular GSH peroxidase could likewise be taken up by the cell for the regeneration of GSH.

The detection of extracellular GST, GSH peroxidase, and homoglutathione raises the question about the physiological function of this system in the apoplast. It is well documented that GST enzymes in higher plants may be

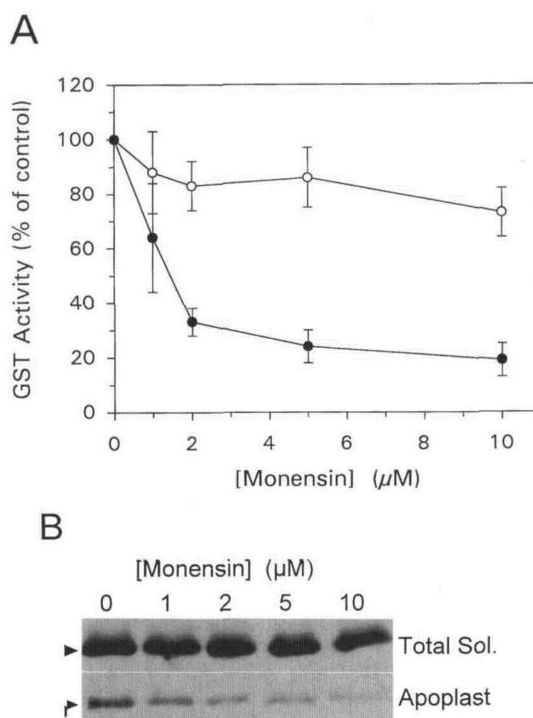

Figure 3. Effect of monensin on the accumulation of apoplastic GST in soybean hypocotyls. Plants were treated with 100 μM TIBA plus the indicated concentrations of monensin for 24 h prior to the preparation of total soluble and apoplastic fractions. A, GST enzyme activity in the total soluble fraction (○) and in the apoplastic fraction (●). Bars indicate SD (n = 3). B, Immunoblot analysis of GST following SDS-PAGE of proteins of the total soluble fraction (Total Sol.) and of the apoplastic fraction (Apoplast). Arrowheads indicate the estimated molecular mass of 26 kD.

induced by a variety of xenobiotics, fungal pathogens and elicitors, wounding, and phytohormones (Dudler et al., 1991; Edwards and Dixon, 1991; Fuerst et al., 1993; Farago et al., 1994; Hahn and Strittmatter, 1994; Flury et al., 1995; Ulmasov et al., 1995). Furthermore, recent studies indicated a protective role of plant GSTs against oxidative stress. In soybean cells, exogenously applied H_2O_2 induces an accumulation of GST transcripts (Tenhaken et al., 1995). Moreover, transient accumulation of H_2O_2 at the plant cell surface in response to attempted pathogen infection or upon exposure of cells to fungal elicitors has been shown to mediate GST mRNA induction. It has been proposed that GST could be involved in the spatial limitation of hypersensitive cell death, which is triggered by an oxidative burst following pathogen ingress (Tenhaken et al., 1995). In animals, the various physiological GST substrates include secondary products of membrane lipid peroxidation, such as α,β-unsaturated aldehydes (Berhane et al., 1994). These findings, along with the known induction of plant GSTs by factors that cause oxidative membrane damage such as wounding, pathogen attack, senescence, and certain xenobiotics, strongly suggest a function of plant GST isoenzymes against oxidative stress and/or in plant-pathogen interactions. Preliminary results from our laboratory indicate that induction of extracellular GST by TIBA in soybean is mediated by an oxidative signal. It will be interesting to elucidate the molecular properties of apoplastic GST/GSH peroxidase and the mechanism of its induction.

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