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Substrate and thiol specificity of a stress-inducible glutathione transferase from soybean

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Abstract An RT-PCR-derived clone encoding a stress-inducible glutathione transferase (GST*Gm*1) from soybean has been over-expressed in *E. coli*. The enzyme was active as the dimer GST*Gm*1-1 and showed GST and glutathione peroxidase activity toward diverse xenobiotics, including analogues of natural stress-metabolites. The selective herbicides, fomesafen and acifluorfen, were conjugated more actively with homoglutathione (hGSH), the major thiol in soybean, than with glutathione (GSH). No thiol preference was shown with the related herbicide, fluorodifen, while GSH was preferred with metolachlor and most non-herbicide substrates. Similar thiol-dependent specificities were observed in GST preparations from plants of varying GSH/hGSH content.

Key words: Glutathione transferase; Herbicides; Glutathione; Glutathione peroxidase; Homoglutathione; (*Glycine max*)

1. Introduction

Glutathione transferases (GSTs, EC 2.5.1.18) catalyse the detoxification of a diverse range of electrophilic compounds by conjugation with glutathione (GSH, γ-glutamyl-L-cysteinyl-L-glycine) [1]. As is the case in animals, plants contain multiple GST isoenzymes, each composed of two subunits, with each type of subunit encoded by a distinct gene [2]. Recently, there has been considerable interest in plant GSTs as they accumulate during infection, chemical injury, senescence and cell division [2]. Although GSTs in plants such as maize (*Zea mays* L.) have defined roles in herbicide and anthocyanin metabolism [2], their function in stress tolerance in other species is unclear.

Soybean (*Glycine max* L.) is known to contain multiple GSTs [3], but their role in herbicide detoxification and endogenous metabolism has not been defined. A number of herbicides which are used to control weeds selectively in soybean are rapidly detoxified by conjugation with homoglutathione [4], as shown in Fig. 1. In soybean, homoglutathione (hGSH, γ-glutamyl-L-cysteinyl-β-alanine) is used instead of glutathione as it is the predominant thiol in this legume species [5]. However, the importance of GSTs in catalysing these conjugations has not been defined. A GST with activity toward 1-chloro-2,4-dinitrobenzene (CDNB) and metolachlor, but low activities toward other herbicides, has been purified from soybean seedlings treated with 2,4-dichlorophenoxyacetic acid [6]. Also, a distinct GST with activity toward CDNB, but of unknown activity toward other substrates, is encoded by a gene independently described as *GH2/4* [7] and *Gmhsp-26a* [8]. The gene has more recently been termed *GmGST26-A* as the polypeptide predicted from its nucleotide sequence has an M_r of 26 kDa [2]. However, we propose to call this gene GST *Glycine max* 1 (*GSTGm1*) as the predicted M_r of GSTs can differ significantly from the M_r of the polypeptides determined by SDS-PAGE. The transcription of the *GSTGm1* gene is increased by heat shock and exposure to heavy metals and a diverse range of xenobiotics [7,8], suggesting that the enzyme has a protective function. Also, because soybean contains hGSH rather than GSH [3] there is a question of the thiol-specificity of GST*Gm*1. The specificity of GSTs toward thiols has only previously been addressed using mammalian GSTs which have 'evolved' to use GSH [9]. We now report that purified recombinant GST*Gm*1, which is active as the dimer GST*Gm*1-1, has broad-ranging activities as a GST and glutathione peroxidase and that enzyme activities are dependent on the type of thiol used in a substrate-specific manner. The thiol-preference of GSTs from species of differing thiol content has also been determined, to establish whether or not the preferential use of hGSH in conjugation is restricted to hGSH-containing plants.

2. Materials and methods

2.1. Plant material and chemicals

Seeds of all species were obtained as detailed previously [10], except for soybean (*Glycine max*) cv. ICI 297 which were supplied by Zeneca Agrochemicals. Plants were grown for 10 days in an environmental growth chamber [10] and the whole plants harvested and frozen in liquid N_2. Suspension-cultures of soybean (L. Merrill cv. Mandarin) were maintained in the dark, at 25°C in Gamborg B5 minimal medium supplemented with sucrose (20 $g \cdot l^{-1}$) and 2,4-dichlorophenoxyacetic acid (1 $mg \cdot l^{-1}$) [11]. Cells were harvested on nylon mesh filters under vacuum, weighed and frozen in liquid N_2. Plant tissue was stored at −80°C prior to use.

Analytical grade (99% pure) herbicides were obtained from Greyhound Chem Service Inc. (Birkenhead, Merseyside L43 4XF), except fomesafen, which was supplied by Zeneca Agrochemicals. *S*-Hexylglutathione was synthesised as described previously [10] and published procedures used to prepare linoleic acid hydroperoxide (13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid) and phosphatidylcholine hydroperoxide to final purities of 90 and 83%, respectively [12]. hGSH was prepared by Zeneca Agrochemicals (85% purity) as described by Adang et al. [9] and references therein.

2.2. Cloning of GSTS1

Total RNA was prepared from soybean cell culture tissue [13] and used as a template for first strand synthesis using AMV reverse transcriptase according to the manufacturer's protocol (Promega). The primer OG2 (5′ GAGAGAGGATCCTCGAGT_{17} 3′) was utilised to selectively synthesise first strand cDNA from polyadenylated mRNA, which served as a template for PCR amplification of *GSTGm1* using

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; GSH, glutathione; GST, glutathione transferase; hGSH, homoglutathione; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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Taq DNA polymerase as recommended by the manufacturer (Gibco BRL). Specific amplification was achieved using GST2 (5′ ATGGCAGCTACTCAGGAAGATGTG 3′), a primer designed towards the previously published 5′ coding sequence of *GSTGm1* [10] and a complementary primer to OG2, OG9 (5′ CGCACTGAGAGAGGATCCTCGAG 3′). The single amplification product was cloned into pGEM-T (Promega) and its identity as *GSTGm1* confirmed by sequencing both strands using an Applied Biosystems 373 DNA Sequencer. To express *GSTGm1* in the desired pET vector (Novagen), an *Nco* I site was introduced at the translational start site using a further primer, GST2-*Nco* (5′ GCGCCATGGCAGCTACTCAGG 3′) and OG9 to amplify from the initial pGEM-T clone. Following cloning of this amplification product into pGEM-T, a *Nco* I/*Bam* HI fragment was purified and cloned into identically digested pET 11d, to create pET-GST*Gm*1. The sequence of pET-GST*Gm*1 was confirmed prior to expression.

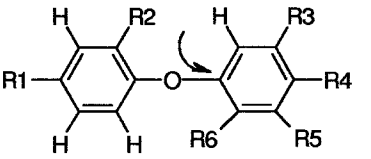
2.3. Expression of GSTGm1

BL21(DE3) bacteria, harbouring pET-GST*Gm*1, were used to inoculate 100 ml of 2X YT media [14] containing 50 μg ml^{-1} carbenicillin, and incubated at 37°C on a shaking platform. After growth to a turbidity of $OD_{600} = 0.5$, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and following a 3 h incubation, the cells were harvested by centrifugation. Bacteria were resuspended in 10 ml of ice-cold 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol (DTT) and then lysed by sonication. After centrifugation (17000×*g*, 20 min) the supernatant was loaded onto a pre-equilibrated column (5 ml) of *S*-hexyl-glutathione linked to Sepharose 6B [10] at 4°C using a Pharmacia GradiFrac apparatus. After washing the column with 10 mM potassium phosphate, pH 7.4, containing 1 mM DTT and 200 mM KCl, GST*Gm*1-containing enzymes were recovered from the column by the addition of 5 mM *S*-hexyl-glutathione. After dialysis for 16 h against 20 mM potassium phosphate, pH 7.4, containing 1 mM DTT, the sample was loaded on a 1 ml HiTrap Q cartridge (Pharmacia) and the enzyme eluted with an increasing concentration of NaCl (0–0.5 M NaCl, total vol 50 ml). At all stages the purification of the recombinant protein was monitored by analysing the samples by SDS-PAGE on 0.8 mm thick gels (12.5% acrylamide and 0.33% *N*′,*N*′-bis-methylene-acrylamide) using a Bio-Rad Mini-Protean apparatus as recommended by the manufacturer.

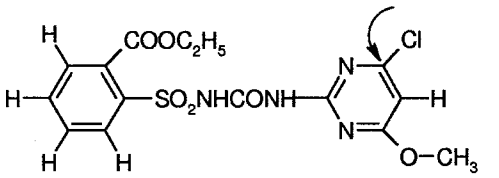
2.4. Enzyme assays

For the assay of plant material, combined roots and foliage were extracted and ammonium sulphate protein precipitates prepared as described previously [10]. Prior to performing assays, enzyme extracts were desalted [10] and protein contents determined using a dye-binding assay with gamma-globulin as the reference protein as recommended by the manufacturer (Bio-Rad). After standardising protein content, spectrophotometric assays were used to determine GST activities toward non-herbicides [10,15] and glutathione peroxidase activity [10].

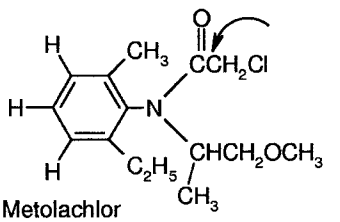
For GST assays with the herbicides, incubations consisted of 10 μl of 10 mM herbicide dissolved in either acetone (chlorimuron ethyl and fluorodifen) or methanol (fomesafen, metolachlor and acifluorfen), 20 μl of 100 mM GSH or hGSH and 120 μl of crude enzyme extract, or purified recombinant enzyme composed of GST*Gm*1 subunits, made up to a final volume of 200 μl with the relevant buffer. With fomesafen, acifluorfen and chlorimuron ethyl the buffer used was 0.4 M Tris-HCl, pH 9.5, for metolachlor 0.1 M potassium phosphate, pH 6.8, and for fluorodifen 0.4 M Tris-HCl, pH 8.5. In all cases the assays were initiated with the addition of GSH or hGSH and the samples incubated at 37°C for 1 h. After stopping the reaction 50 μl of the reaction mixture was analysed by reversed-phase HPLC as described previously [10]. GSH and hGSH conjugates were identified and quantified with reference to authentic standards prepared by reacting the herbicide with GSH, or hGSH, at alkaline pH [10]. The retention times of the conjugates are given in Table 1. In all cases enzyme activities were expressed in katals after correcting for the reaction rate in the absence of enzyme (Table 1).



Acifluorfen. R1=CF_3, R2=Cl, R3=COOH, R4=NO_2.
Fluorodifen. R1=NO_2, R4=CF_3, R6=NO_2.
Fomesafen. R1=CF_3, R2=Cl, R4=NO_2, R5=$CONHSO_2CH_3$.



Chlorimuron Ethyl



Metolachlor

Fig. 1. Structures of herbicides referred to in the text, with the position of nucleophilic substitution with hGSH shown with an arrow.

Table 1
Non-enzymic conjugation rates of GSH and hGSH with herbicides and retention times of products as determined by HPLC

Herbicide	Thiol	Non-enzymic rate		Retention time (min)
		pkat	(assay pH)	
Acifluorfen	GSH	ND	(9.5)	10.7
	hGSH	ND	(9.5)	11.2
Chlorimuron ethyl	GSH	ND	(9.5)	19.5
	hGSH	ND	(9.5)	19.6
Fluorodifen	GSH	0.7	(8.5)	17.3
	hGSH	0.7	(8.5)	17.5
Fomesafen	GSH	ND	(9.5)	10.7
	hGSH	ND	(9.5)	11.1
Metolachlor	GSH	1.1	(6.8)	18.5
	hGSH	1.0	(6.8)	18.7

ND = not detected. Non-enzymic reaction rates were determined at the pH used to measure the enzymic rate with the respective herbicide.

Table 2
GST and glutathione peroxidase activities of the recombinant GST*Gm*1-1 enzyme toward a variety of substrates in the presence of GSH and hGSH

Substrate	Enzyme activity	
	GSH	hGSH
GST activity	*(nkat·mg^{-1} protein) – Non-herbicides*	
Benzyl isothiocyanate	31.8 ± 9.2	15.0 ± 2.0
CDNB	121.8 ± 2.4	65.4 ± 3.0
Ethacrynic acid	3.7 ± 1.4	1.0 ± 0.3
1,2-Epoxy-3-(*p*-nitro-phenoxypropane)	0.4 ± 0.3	0.2 ± 0.0
p-Nitrobenzyl chloride	0.9 ± 0.1	0.5 ± 0.1
4-Vinylpyridine	0.5 ± 0.2	0.1 ± 0.1
GST activity	*(pkat·mg^{-1} protein) – Herbicides*	
Acifluorfen	12.9 ± 0.3	42.0 ± 2.0
Fomesafen	27.9 ± 1.9	138.5 ± 6.9
Fluorodifen	396.9 ± 17.9	443.1 ± 10.9
Chlorimuron ethyl	9.8 ± 1.6	16.3 ± 0.9
Metolachlor	228.3 ± 18.9	91.9 ± 16.1
Glutathione peroxidase activity (OD change at 366 nm·min^{-1}·mg^{-1} protein)		
Cumene hydroperoxide	0.44 ± 0.13	0.50 ± 0.15
Linoleic acid hydroperoxide	0.08 ± 0.03	0.08 ± 0.02
Phosphatidylcholine hydroperoxide	ND	ND

Each value represents the mean of 2 replicates ± the variation between the replicates and the mean. ND = none detected.

3. Results

A full-length cDNA clone of *GSTGm1* was derived from RT-PCR of RNA isolated from 5-day-old soybean cell cultures. An identical amplification product could also be obtained using the RNA from 5-day-old soybean seedlings, confirming that mRNA encoding GST*Gm*1 is found in both cell cultures and plants. Using the existing numbering system, when compared with the published genomic sequence of *Gmhsp26-a* [8], two nucleotide substitutions were observed in the coding sequence of the RT-PCR clone at position 276 (A to G) and position 1042 (C to T). The substitutions in the RT-PCR clone did not introduce any changes in the respective amino acid sequence and the recombinant GST*Gm*1 was considered to be identical to the expected enzyme in soybean. The sequence of the RT-PCR clone was compared with that of the partial sequence originally described for the auxin-inducible transcript from soybean, *GH2/4* [16]. Three nucleotide substitutions were observed in the coding sequence, together with both a deletion and substitution in the 3′-untranslated region. The substitution at position 1042 resulted in a single amino acid change, and was also observed in *Gmhsp-26a* [8]. These minor changes could have arisen from PCR-derived errors; however, this seems unlikely as identical differences in the DNA sequence of *GH2/4* and *Gmhsp-26a* have been noted previously and ascribed to differences in the cultivar of soybean used [7].

GST*Gm*1 was expressed in *E. coli* and the respective functional GST purified using affinity chromatography and Q-sepharose anion exchange chromatography [10]. When the purified native enzyme was analysed by gel filtration chromatography, it co-eluted with ovalbumin, with an M_r of 45 kDa being determined [10]. This confirmed that the GST was active as the dimer GST*Gm*1-1. When analysed by SDS-PAGE, the GST*Gm*1 subunit migrated as a 29 kDa polypeptide, 3 kDa larger than the predicted M_r. Similarly, we have observed that the subunits of other plant GSTs migrate as larger polypeptides than predicted from the nucleotide se-

Table 3
Usage of GSH and hGSH in the conjugation of the herbicides fomesafen, fluorodifen and metolachlor in plants of differing thiol composition

Species	Thiol	% Thiol abundance	Enzyme activity (pkat·mg^{-1})		
			Fomesafen	Fluorodifen	Metolachlor
G. max	GSH	1%	ND	0.21 ± 0.02	1.67 ± 0.32
	hGSH	99%	0.73 ± 0.03	0.38 ± 0.05	0.92 ± 0.25
P. vulgaris	GSH	1%	ND	0.52 ± 0.02	1.35 ± 0.66
	hGSH	99%	3.68 ± 0.18	0.79 ± 0.03	0.66 ± 0.03
M. sativa	GSH	34%	ND	0.25 ± 0.00	0.41 ± 0.01
	hGSH	66%	0.18 ± 0.00	0.20 ± 0.06	ND
T. pratense	GSH	24%	ND	0.76 ± 0.03	0.50 ± 0.05
	hGSH	76%	0.28 ± 0.02	0.49 ± 0.06	ND
P. sativum	GSH	99%	ND	0.79 ± 0.02	0.21 ± 0.02
	hGSH	1%	ND	0.42 ± 0.08	ND
Z. mays	GSH	100%	ND	0.43 ± 0.06	2.21 ± 0.07
	hGSH	ND	0.35 ± 0.02	0.22 ± 0.03	1.18 ± 0.00
E. crus-galli	GSH	100%	0.06 ± 0.00	0.51 ± 0.01	ND
	hGSH	ND	0.16 ± 0.00	0.20 ± 0.02	ND

Values refer to the means of 2 replicates, each of multiple plants ± the variation between the replicates and the mean. ND = not detected. The relative thiol contents refer to previously published data [5,19,20].

quence when analysed by SDS-PAGE [17]. GST*Gm*1-1 was assayed for GST and glutathione peroxidase activities with a range of substrates using both GSH and hGSH (Table 2). All assays were run at saturating substrate concentrations under conditions where product formation was strictly dependent on incubation time and protein content. With the non-herbicides, GST activities were determined with a diverse range of substrates, with low activities (< 0.1 nkat.mg^{-1} pure protein) also being determined with bromosulphophthalein, 1,2-dichloro-4-nitrobenzene, *trans*-4-phenyl-3-buten-2-one and *p*-nitrophenethyl bromide. With all these substrates GST activity was at least two-fold higher with GSH, rather than hGSH. However, in the absence of enzyme, the respective rates of conjugation were similar with both thiols (data not shown). With the herbicides a substrate-dependent preference was seen in thiol usage. The diphenyl ether herbicides, acifluorfen and fomesafen, were more actively conjugated in the presence of hGSH rather than GSH, while fluorodifen was conjugated equally well with either thiol. Similarly, with chlorimuron ethyl, conjugation proceeded more readily with hGSH. In contrast, GST activity toward metolachlor was over two-fold higher with GSH rather than hGSH. As determined with the non-herbicide substrates, differences in reaction rates with GSH and hGSH could not be accounted for by variations in thiol reactivity, as GSH and hGSH gave similar non-enzymic conjugation rates with each herbicide (Table 1).

GST*Gm*1-1 was also assayed for glutathione peroxidase activity, as several soybean GSTs have been reported to have this secondary activity [3]. Pure GST*Gm*1-1 used both hGSH and GSH equally efficiently to reduce cumene hydroperoxide and linoleic acid hydroperoxide. As expected, no activity was determined with phosphatidylcholine hydroperoxide, which is a substrate for selenium-dependent glutathione peroxidases [18].

Having determined that a soybean GST showed a substrate-dependent preference for hGSH, it was then of interest to determine whether this selective thiol usage was shown in the GSTs from plants of varying GSH/hGSH content. Species were selected which contained predominantly hGSH, soybean and French bean (*Phaseolus vulgaris* L.) [5], predominantly GSH, pea (*Pisum sativum* L.) [5] and maize [19], and a mixture of GSH and hGSH, alfalfa (*Medicago sativa* L.) [20] and red clover (*Trifolium pratense* L.) [5]. The weed, barnyard grass (*Echinochloa crus-galli* L.), was also included as preliminary studies had shown that it could detoxify fomesafen. HPLC analysis [20] confirmed that barnyard grass contained GSH as its major thiol, with no hGSH being detected. To test for thiol-dependence in the various species, GST activity was determined toward fomesafen, fluorodifen and metolachlor (Table 3). With fomesafen as substrate, GST preparations from all species showed a similar preference for hGSH over GSH. Similarly, all GST activities toward metolachlor were greater with GSH than hGSH. With fluorodifen as substrate, in the species containing hGSH only, the crude GST preparations showed a preference for hGSH. In alfalfa, the GSTs used GSH and hGSH equally well, while in the remaining species GSH was the preferred co-substrate.

4. Discussion

Pure, recombinant GST*Gm*1-1 showed GST activities toward a diverse range of xenobiotic substrates, including the herbicides used selectively in soybean (Fig. 1 and Table 2). Although detailed kinetic analyses have not been performed, with the majority of substrates, GST*Gm*1-1 behaved in a similar manner to that described for mammalian GSTs, namely that GSH was preferred over hGSH as thiol co-substrate [9]. However, with the diphenyl ether herbicides, particularly acifluorfen and fomesafen, and the sulphonyl urea, chlorimuron ethyl (Fig. 1), this preference was reversed. This suggested that GST*Gm*1-1 may have 'evolved' to use selectively the endogenous thiol hGSH more efficiently than GSH, with the resulting rapid detoxification of the herbicides protecting soybean from phytotoxic injury. However, this simplistic conclusion proved incorrect, as GST activities toward fomesafen were also higher with hGSH in plants of differing thiol composition (Table 3). However, there was a suggestion of subtle adaptations in the GSTs of hGSH-containing plants, as the crude enzymes in French bean and soybean catalysed the conjugation of fluorodifen with hGSH relatively more effectively than with GSH, while the opposite was true of the enzymes from GSH-containing plants. The strong preference for hGSH in the conjugation of fomesafen and acifluorfen warrants further study. There was no evidence that the SH groups of hGSH and GSH differ in their reactivities (Table 1), confirming earlier observations [9], and this would suggest that subtle variations in size between the two types of glutathione must cause a closer co-ordination between hGSH and fomesafen/acifluorfen at the active site of GSTs than is possible with GSH and the herbicides. It will now be of interest to examine this possibility in greater detail and define the specificity of GST*Gm*1-1 for GSH and hGSH by determining the ratio of k_{cat}/K_m for these thiols. However, whatever the mechanism of preferential hGSH usage, it is possible that the reason that fomesafen and acifluorfen are selective in soybean is that this species contains hGSH, rather than GSH.

The *GSTGm1* gene is activated by exposure to heat shock [8], heavy metals and a range of auxins and non-auxin analogues [7]. Our studies have revealed that GST*Gm*1-1 can detoxify ethacrynic acid and vinyl pyridine, compounds which are structurally analogous to propenal derivatives known to accumulate during oxidative damage to lipids and nucleic acids [10]. In addition, GST*Gm*1-1 had activity as a glutathione peroxidase, capable of reducing toxic linoleic acid hydroperoxide, a major stress-induced reaction product formed in soybean by the action of lipoxygenases [12], to the corresponding alcohol. The stress-inducible GST*Gm*1-1 may therefore serve to detoxify both naturally-occurring propenals and fatty acid hydroperoxides which accumulate as a result of oxidative stress imposed by infection, chemical injury and heat shock [3]. Although a constitutively-expressed GST with glutathione peroxidase activity has been described in *Arabidopsis*, the effect of stress-treatment on its expression was not reported [21]. Consistent with our proposal that stress-inducible GSTs detoxify oxidised natural products, recent studies from our laboratory have shown that glutathione peroxidase activity is increased in the roots of peas treated with heavy metals [10]. It will now be of interest to determine how many of the stress-inducible GSTs reported in plants [2] have GST and glutathione peroxidase activities directed toward the products of oxidative stress.

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