

Influence of Chemical Treatments on Glutathione S-Transferases of Maize with Activity Towards Metolachlor and Cinnamic Acid

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Z. Naturforsch. **53c**, 973–979 (1998); received June 8/July 20, 1998

Benoxacor, Cinnamic Acid, Glutathione S-Transferase, Maize, Metolachlor, Safener

The subcellular distribution of glutathione S-transferase (GST) activity extracted from shoots of 3-day-old etiolated seedlings of maize (*Zea mays* L., Northrup-King 9283 hybrid) and the induction of soluble and membrane-bound GST activity by the safener benoxacor, the herbicide metolachlor and their combination (CGA-180937) were investigated. GST activity extracted from maize shoots was detected in both cytosolic and microsomal fractions and utilized 1-chloro-2,4-dinitrobenzene (CDNB), metolachlor, and *trans*-cinnamic acid (CA) as substrates. Soluble GST activity extracted from maize shoots was greater than microsomal with CDNB or metolachlor as substrate. Membrane-bound GST activity was greater than soluble with cinnamic acid as substrate. Washing the microsomal preparations from maize shoots with Triton X-100 increased GST(CA) activity. Pretreatment with the safener benoxacor or a formulated combination of the herbicide metolachlor with benoxacor induced soluble GST(CDNB), GST(metolachlor) and GST(CA) activities in maize shoots. Benoxacor and CGA-180937 induced also membrane-bound GST(CDNB) and GST(CA) activities in maize shoots, but did not affect membrane-bound GST(metolachlor) activity. These results confirm that maize contains multiple GST isozymes that differ in their substrate specificity and inducibility by safeners or other chemicals.

Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of dimeric multifunctional enzymes catalyzing the conjugation of the thiol group of glutathione to various electrophilic substrates (Cole *et al.*, 1997; Daniel, 1993; Marrs, 1996). Although the endogenous roles of plant GSTs are still poorly understood, their involvement in cellular protection against oxidative damage and as carrier proteins for auxins has been proposed (Alfen-

ito and Walbot, 1997; Kömives *et al.*, 1997; Marrs, 1996). In addition to their suggested endogenous functions, plant GSTs play a well established role in the metabolic detoxification of several herbicides and other xenobiotic chemicals (Cole *et al.*, 1987; Marrs, 1996).

Although primarily described as cytosolic enzymes, membrane-bound GSTs have been described from rat liver (Mannervik and Danielson, 1988) and plants (Diesperger and Sandermann, 1979; Edwards and Dixon, 1991). The microsomal GSTs have been associated with the conjugation of endogenous substrates such as cinnamic acid and are of particular interest in defining a biological role for plant GSTs (Dean and Machota, 1993; Dean *et al.*, 1995; Diesperger and Sandermann, 1979; Edwards and Dixon, 1991).

The tolerance of maize and other grass plants to chloroacetanilide herbicides such as metolachlor results from a GST-mediated detoxification of these herbicides (Gronwald, 1989; Timmerman, 1989). Much research has focused on the enhancement of this tolerance by herbicide safeners (Fargo *et al.*, 1994; Gronwald, 1989; Hatzios, 1991).

Abbreviations: Benoxacor, 4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine; CA, *trans*-cinnamic acid; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione S-transferase; GST(CA); GST activity measured with *trans*-cinnamic acid as substrate; GST(CDNB), GST activity measured with CDNB as substrate; GST(metolachlor), GST activity measured with metolachlor as substrate; metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-acetamide; Rf, ratio of front; TLC, thin-layer chromatography.

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0939-5075/98/1100-0973 \$ 06.00 © 1998 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com. D



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The safener benoxacor is used commercially to protect maize against injury from the herbicide metolachlor (Cottingham and Hatzios, 1991; Kreuz *et al.*, 1991; Miller *et al.*, 1993; Viger *et al.* 1991). Benoxacor and other safeners stimulate GST activity, and in some cases induce novel GST isozymes with specific specificity for herbicidal substrates (Dean *et al.* 1990 and 1991; Fuerst *et al.*, 1993; Irzyk and Fuerst, 1993 and 1997; Jepson *et al.*, 1994; Mozer *et al.*, 1983; Wiegand *et al.*, 1986). Several GST isozymes have been described in plants, including seven well-characterized isozymes from maize that can be distinguished by their biochemical and molecular characteristics, substrate specificities and inducibility by safeners (Irzyk and Fuerst, 1997; Marrs, 1996; Frova *et al.* 1997).

The purpose of this study was to characterize the subcellular localization of maize GST activity responsible for the metabolism of endogenous and herbicidal substrates and the regulation of GST activity by the chemical treatments. Our specific objectives were to: a) characterize the specificity of soluble and microsomal GST activity extracted from the maize hybrid 'Northrup-King 9283' using CDNB, metolachlor, and *trans*-cinnamic acid as substrates; b) determine the effects of the safener benoxacor, the herbicide metolachlor, and the combination of metolachlor and benoxacor (CGA-180937) on maize GST activity utilizing the aforementioned substrates.

Materials and Methods

Chemicals

The chemical structures of the three GST substrates used in this study as well as of the safener benoxacor are shown in Fig. 1. Analytical grade (95–99% pure) samples of benoxacor and *rac*-metolachlor, technical grade sample of CGA-180937 (mixture of metolachlor and benoxacor at 30:1, w/w) and radiolabeled sample of metolachlor [carbonyl-labeled ^{14}C , sp. act. act. 7.77 MBq/mg] were provided by Novartis Crop Protection, Basel, Switzerland. [(Side-chain-3- ^{14}C)cinnamic acid, sp. act. act. 14.1 MBq/mg] was purchased from Amersham Corporation, Arlington Heights, Illinois, USA. Solvents and other reagents (*e.g.*, GSH and CDNB) were obtained commercially from Sigma,

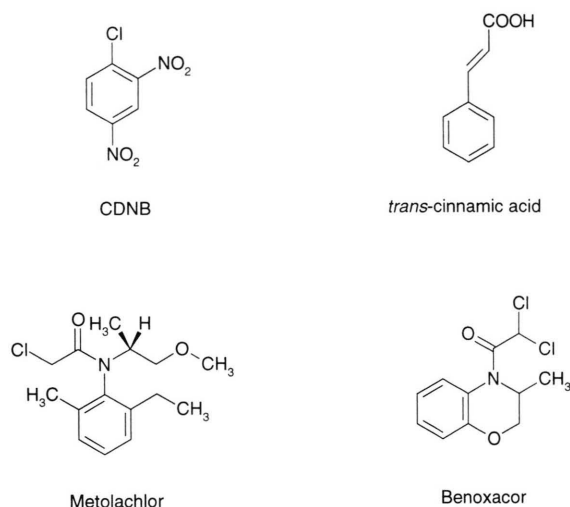


Fig. 1. Chemical structures of the three GST substrates and of the safener benoxacor.

St. Louis, Missouri, USA, and Fisher, Pittsburgh, Pennsylvania, USA.

Plant material and chemical treatments

Seeds of 'Northrup-King 9283' corn maize were rinsed for 5 min with tepid tap water, blotted dry, and placed on three layers of paper towels in 20 x 20 cm plastic containers with tight fitting lids. The paper towels were saturated with distilled water (150 ml) and seeds were allowed to germinate for 72 hr in a dark growth chamber at 30 °C. To determine the influence of benoxacor, metolachlor, or their formulated combination (CGA-180937) on GST activity, maize seeds were prepared as above with the inclusion of 1 μM benoxacor, 30 μM metolachlor, or 30 μM CGA-180937 in the imbibing solution. Etiolated maize seedlings were 3-day old at harvest time and used in all subsequent studies.

Extraction of soluble and microsomal GST

Crude extracts of GST were obtained from soluble and microsomal fractions of maize tissues. Microsomes from shoots of 3-day old etiolated maize seedlings were prepared according to Mcfadden *et al.* (1990) with slight modification. All steps were conducted at 4 °C. Two grams of excised shoots were frozen with liquid nitrogen and ground to a fine powder in a mortar. The ground tissues were then homogenized in 5 vol-

umes of an ice-cold medium containing 200 mM Tris-HCl (tris[hydroxymethyl]aminomethane hydrochloride, pH 7.8), 25 mM mercaptoethanol, 1 mM EDTA and 10% (w/v) glycerol. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, California, USA) and then centrifuged for 90 min at 100,000×g. The supernatant of this centrifugation was used for the determination of soluble GST activity and protein content. The remaining pellet (microsomal fraction) was resuspended in 1 ml of the homogenization buffer medium (Tris-HCl) supplemented with or without 0.1% Triton X-100 (Calbiochem, La Jolla, California, USA) and centrifuged again at 100,000×g for 90 min at 4 °C. Supernatants of this last centrifugation were used to assay membrane-bound GST activity and the effect of Triton X-100 on microsomal GST(CA) activity.

Assays of GST activity

GST activity was assayed using CDNB, metolachlor, and *trans*-cinnamic acid as substrates. CDNB-specific GST activity in soluble and microsomal extracts from unsafened, benoxacor-treated (1 µM), metolachlor-treated (30 µM) or CGA-180937-treated (30 µM) maize tissues was determined according to Mannervik and Guthenberg (1981). The reaction mixture contained 30 µl of the enzyme extract, 2 ml of 100 mM potassium phosphate buffer (pH 6.9), 0.9 ml of 3.3 mM GSH, and 100 µl of 30 mM CDNB. The mixture was incubated at room temperature (25 °C), and the reaction was started by the addition of CDNB. The change in absorbance due to the formation of GS-CDNB conjugate was measured spectrophotometrically at 340 nm. GST activity was calculated and corrected for nonenzymatic conjugation according to Mannervik and Guthenberg (1981).

GST(metolachlor) activity in soluble and microsomal extracts from unsafened, benoxacor-safened (1 µM), metolachlor-treated (30 µM) and CGA-180937-treated (30 µM) maize shoots were determined by the method of Mozer *et al.* (1983) with slight modifications. GST(CA) activity in maize shoot extracts was determined by the method of Edwards and Dixon (1991). The 70-µl reaction mixture for the GST(metolachlor) and GST(CA) assays contained 20 µl of soluble or microsomal enzyme extract, 30 µl of 100 mM phosphate buffer

(pH 6.9), 10 µl of 60 mM GSH, and 10 µl of 100 mM of [¹⁴C]metolachlor or [¹⁴C]*trans*-cinnamic acid (CA) (481 Bq). The reaction was initiated by the addition of the radiolabeled substrate and the mixture was incubated for 60 min at 30 °C. The reaction was stopped by the addition of 60 µl of 5% trichloroacetic acid. The reaction mixture was then partitioned with 1 ml of dichloromethane to remove the unmetabolized substrate. An aliquot of the aqueous phase was removed and the quantity of ¹⁴C in the formed glutathione conjugates was determined by liquid scintillation counting. The GS-conjugate of metolachlor was confirmed by co-chromatography with a synthetic conjugate on TLC plates that were developed in a chloroform:methanol:formic acid:water (75:25:4:2, v/v/v). The glutathione and cysteine conjugates of cinnamic acid were detected by TLC using butanol:acetic acid:water (12:3:5, v/v/v) as developing system (Edwards and Dixon, 1991). In all assays, reported GST activity values were corrected for nonenzymatic conjugation. Each treatment was replicated three times and all experiments were repeated.

Protein concentration in tissue extracts was determined spectrophotometrically at 280 nm according to the method of Bradford (1976) using BSA as a protein standard.

Results

Soluble and membrane-bound GST activity in maize seedlings

GST activity detected in maize shoots was both soluble and membrane-bound (Tables I and IV). The subcellular distribution of GST activity revealed a substantial membrane-bound GST(metolachlor) activity (Table I). The membrane-bound GST(metolachlor) activity demonstrated here represents only a fraction of the total GST(metolachlor) activity (about 15%). The potential involvement of the membrane-bound GST(metolachlor) activity in the native tolerance of maize to metolachlor is not clearly understood at the present time. Wu *et al.* (1996) have demonstrated also the presence of membrane-bound (microsomal) GST activity utilizing the glutathione conjugation of the herbicide pretilachlor in shoot and root tissues of rice. It would be interesting to determine if susceptible weed species possess a similar mem-

Table I. Subcellular distribution of GST(metolachlor) activity in shoots of 3-day old 'Northrup-King 9283' maize seedlings.

Cell fraction	GST(metolachlor) activity	
	(pmol/min x mg)	Total ^a
Soluble	33.3 ± 2.7	464 ± 9.8
Membrane-bound	23.2 ± 2.1	74 ± 3.7

^a Total units of GST activity calculated by multiplying enzyme activity, expressed as pmol GS-metolachlor conjugate formed per min per mg protein, times total protein in respective fraction. Data represent the average and standard errors of two experiments with 3 replications.

brane-bound GST(metolachlor) activity. Such a study might provide further insight on the basis of metolachlor selectivity.

Shoot extracts of Northrup-King 9283 maize were evaluated for GST activity utilizing [¹⁴C]*trans*-cinnamic acid as substrate. Following the incubation of soluble and microsomal GST preparations with radiolabeled cinnamic acid and glutathione (GSH) for 60 min, thin-layer chromatography (TLC) analysis revealed three major metabolites which were consistent with those observed by Edwards and Dixon (1991). Based on their relative mobilities, these products appear to correspond to the glutathione-conjugate of *trans*-cinnamic acid (Rf 0.16), the cysteine-conjugate of *trans*-cinnamic acid (Rf 0.38) and an unidentified metabolite of *trans*-cinnamic acid (Rf 0.63), (Table II). A small portion (2.25 to 2.5%) of the radioactivity recovered by TLC analysis remained near the origin. The formation of glutathione and cysteine conjugates of *trans*-cinnamic acid in microsomal extracts was 2.38-fold and 3.74-fold greater than when soluble extracts were used in these reactions (Table II).

These results are in agreement with previous reports demonstrating membrane-bound GST activity in monocot and dicot plants. Membrane-bound (microsomal) GST activity was first described from cell cultures of *Phaseolus vulgaris* and *Pisum sativum* (Diesperger and Sandermann, 1979). These preparations were quite active in catalyzing the conjugation of cinnamic acid with GSH but did not catalyze the conjugation of metolachlor with glutathione. Similarly, microsomal fractions from cell cultures of Black Mexican sweetcorn (*Zea mays* L.) were reported to have glutathione *S*-cinnamoyl transferase activity but were not tested for GST(metolachlor) activity (Dixon and Edwards, 1991).

Effect of Triton X-100 on Glutathione S-cinnamoyl transferase activity of maize

Washing the preparations with the bound GST with 0.1% Triton X-100 caused a significant increase in glutathione *S*-cinnamoyl activity as indicated by the 1.61-fold increase in the amount of ¹⁴C-label recovered as the GS-conjugate of cinnamic acid (Table III). Detergent treatment may be removing the peptidase responsible for the catalysis of the GS-conjugate to the cysteine-conjugate, resulting in the observed decrease of that metabolite and the associated increase in the GS-conjugate (Table III). Interestingly, treatment with Triton X-100 caused a significant decrease in the amount of ¹⁴C-label recovered in the cinnamic acid metabolite migrating to Rf 0.63 and an increase in the percent radioactivity recovered at the origin (Table III). This indicates that the enzyme catalyzing this conversion is either a cytosolic contaminant associated weakly with the microsomal preparations used in our studies or sensitive to the detergent Triton X-100.

Table II. Reaction products detected by TLC analysis following the incubation of ¹⁴C-labeled *trans*-cinnamic acid with soluble and membrane-bound extracts from 3-day old etiolated maize shoots.

Cinnamic acid metabolites	Rf value ^a	Soluble fraction (% of recovered ¹⁴ C)	Bound fraction (% of recovered ¹⁴ C)	Bound/Soluble ratio
Origin	0.00	2.50	2.25	0.90
GS-conjugate	0.16	5.25	12.50	2.38
Cys-conjugate	0.38	2.75	10.30	3.74
Unknown	0.63	11.75	15.75	1.34

^a TLC solvent system was butanol:acetic acid:water (12:3:5, v/v/v).

Table III. Effect of Triton X-100 on the metabolism of ^{14}C -labeled *trans*-cinnamic acid by membrane-bound extracts from 3-day old etiolated maize shoots.

Cinnamic acid metabolites	Rf value ^a	Untreated (% of recovered ^{14}C)	Triton X-100 (0.1%) (% of recovered ^{14}C)	Triton/untreated Ratio
Origin	0.00	2.00	8.00	4.00
GS-conjugate	0.16	9.30	15.00	1.61
Cys-conjugate	0.38	15.00	11.70	0.78
Unknown	0.63	14.30	6.30	0.44

^a TLC solvent system was butanol:acetic acid:water (12:3:5, v/v/v).

Effect of benoxacor and metolachlor treatments on maize GST activity

The influence of metolachlor, benoxacor, and the metolachlor/benoxacor mixture (CGA-180937) on soluble and membrane-bound GST activity of maize shoots is shown in Table IV. Similar to previous reports (Timmerman, 1989), GST(CDNB) activity was considerably higher than that of GST(metolachlor) and GST(CA) activities. Soluble GST(CDNB) and GST(metolachlor) activities were enhanced by 18–41% following treatment with 1 μM of benoxacor or 30 μM of CGA-180937 (metolachlor:benoxacor, 30:1 w/w). Treatment with 30 μM metolachlor did not affect soluble GST(metolachlor) activity, but decreased soluble GST(CDNB) activity in maize shoots (Table IV). This does not agree with the results of Dean *et al.* (1990), who reported that treatment with metolachlor at concentrations up to 160 μM stimulated GST(metolachlor) activity in grain sorghum, but had no effect on GST(CDNB) activity.

Membrane-bound GST(metolachlor) activity was not influenced by any of the metolachlor or benoxacor treatments (Table IV) indicating that GST activity in this fraction is distinct and has not

been substantially contaminated by cytosolic GST. In contrast, membrane-bound GST(CDNB) activity was enhanced by 33–50% by all chemical treatments. This suggests that these microsomal preparations contain an inducible GST isozyme which can be measured with CBNB as substrate and may not possess GST(metolachlor) activity.

Membrane-bound GST(CA) activity in extracts from maize shoots appeared to be about 3-fold greater than soluble GST(CA) activity (Table IV). This is in agreement with the results of Edwards and Dixon (1991), who showed also a 3-fold greater microsomal GST(CA) activity in cell extracts of black Mexican sweetcorn. Treatment with metolachlor, benoxacor or their combination (CGA-180937) did not alter soluble GST(CA) activity in maize shoots (Table IV). However, membrane-bound GST(CA) activity was enhanced by 34–42% following treatment with 1 μM of benoxacor or 30 μM of CGA-180937 (Table IV).

Discussion

Overall, the results of the present study confirm the presence of multiple GST activities in maize, which differ in their substrate specificity and inducibility by safeners or other chemical treatments.

Table IV. Soluble and membrane-bound GST activities extracted from shoots of maize seedlings treated with metolachlor, benoxacor, or their combination^a.

Treatment	GST(CDNB) ($\mu\text{mol}/\text{min} \times \text{mg}$)				GST(metolachlor) ($\text{pmol}/\text{min} \times \text{mg}$)				GST(CA) ($\text{nmol}/\text{min} \times \text{mg}$)			
	Soluble	Percent of control	Bound	Percent of control	Soluble	Percent of control	Bound	Percent of control	Soluble	Percent of control	Bound	Percent of control
Control	39.2	100	6.4	100	29.7	100	23.8	100	8.8	100	24.8	100
Metolachlor	29.8	76	8.5	135	31.8	107	25.2	106	8.5	97	23.4	94
Benoxacor	46.2	118	9.1	142	42.0	141	24.8	104	10.1	115	35.2	142
CGA-180937	53.8	137	9.6	150	40.7	137	21.8	92	9.8	111	33.2	134

^a Seeds were imbibed with either 1 μM benoxacor, 30 μM metolachlor, or 30 μM CGA-180937 (metolachlor:benoxacor, 30:1, w/w).

Maize GST isozymes active towards chloracetanilide herbicides and their induction by herbicide safeners have been discussed in several recent publications (Marrs, 1986; Cole *et al.* 1997; Irzyk and Fuerst, 1997; Frova *et al.*, 1997).

In addition to utilizing herbicidal substrates and CDNB, soluble and microsomal extracts of maize GST used in our studies were active with *trans*-cinnamic acid, an endogenous plant metabolite. Most of the GST(CA) activity was associated with membrane-bound (microsomal) rather soluble GST preparations. These results are in agreement with earlier reports confirming that in some plant species (*e.g.*, peas, soybean and maize), glutathione *S*-cinnamoyl transferase may function *in vivo* to produce glutathione conjugates of *trans*-cinnamic acid (Diesperger and Sandermann, 1978; Dixon and Edwards, 1991). Previous reports by Edwards and Owen (1987) and by Dean *et al.* (1991) had suggested that the GST enzyme responsible for the glutathione conjugation of CA is distinct from the enzymes that are active with various herbicides and the general substrate CDNB. More recently Dean and Machota (1993) and Dean *et al.* (1995) have shown that maize shoots may contain several GST isozymes that are active with CA.

It has been suggested that some of these GSTs may be involved in plant defense responses, since

exposure of bean cell suspensions to a fungal elicitor induced GST(CA) activity (Dixon and Edwards, 1991). The maize GST active with CA has been shown to be also active with *p*-coumaric acid and other phenylpropanoids (Dean *et al.*, 1995). Conjugation of these compounds with glutathione may reduce their toxicity and increase their sequestration in the vacuoles of plant cells (Alfenito and Walbot, 1997). Elevated levels of phenylpropanoid compounds during periods of plant stress may regulate the activity of GST(CA). Dean and Machota (1993) have shown that *p*-coumaric acid and 7-hydroxycoumarin can activate GST(CA) in maize shoots. Further studies are necessary to elucidate the biological significance, gene structure and regulation of expression of plant GSTs with activity towards CA and other endogenous compounds.

Acknowledgments

The authors thank Novartis Crop Protection (Formerly Ciba-Geigy) for providing the analytical, radiolabeled, and formulated samples of metolachlor and benoxacor used in this study as well partial financial support. Gratitude is also expressed to Northrup-King for providing seeds of the NK-9283 hybrid of maize.

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