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Partial Characterization of Glutathione *S*-Transferases from Wheat (*Triticum* spp.) and Purification of a Safener-Induced Glutathione *S*-Transferase from *Triticum tauschii*[1]

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Hexaploid wheat (*Triticum aestivum* L.) has very low constitutive glutathione *S*-transferase (GST) activity when assayed with the chloroacetamide herbicide dimethenamid as a substrate, which may account for its low tolerance to dimethenamid in the field. Treatment of seeds with the herbicide safener fluxofenim increased the total GST activity extracted from *T. aestivum* shoots 9-fold when assayed with dimethenamid as a substrate, but had no effect on glutathione levels. Total GST activity in crude protein extracts from *T. aestivum*, *Triticum durum*, and *Triticum tauschii* was separated into several component GST activities by anion-exchange fast-protein liquid chromatography. These activities (isozymes) differed with respect to their activities toward dimethenamid or 1-chloro-2,4-dinitrobenzene as substrates and in their levels of induction by safener treatment. A safener-induced GST isozyme was subsequently purified by anion-exchange and affinity chromatography from etiolated shoots of the diploid wheat species *T. tauschii* (a progenitor of hexaploid wheat) treated with the herbicide safener cloquintocet-mexyl. The isozyme bound to a dimethenamid-affinity column and had a subunit molecular mass of 26 kD based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme (designated GST TSI-1) was recognized by an antiserum raised against a mixture of maize (*Zea mays*) GSTs. Amino acid sequences obtained from protease-digested GST TSI-1 had significant homology with the safener-inducible maize GST V and two auxin-regulated tobacco (*Nicotiana tabacum*) GST isozymes.

GSTs (EC 2.5.1.18) play an important role in xenobiotic detoxification in plants (Lamoureux and Rusness, 1989; Kreuz et al., 1996). Attempts to determine the endogenous functions of plant GSTs have led to their extensive study in recent years. GSTs may have important roles in protection from cytotoxic endogenous compounds such as anthocyanins and phytoalexins (Li et al., 1997), and in regulation, transport, and potential GSH conjugation with auxins (for review, see Marrs, 1996). GSTs also appear to have a role in GSH conjugation with endogenous secondary metabolites such as unsaturated phenylpropanoids (Diesperger and Sandermann, 1979; Edwards and Dixon, 1991; Dean and Machota, 1993; Dean et al., 1995), although more recently this activity in maize (*Zea mays*) has been attributed to an ascorbate peroxidase enzyme (Dean and Devarenne, 1997).

Many plant species contain GST activity when assayed with xenobiotic and potential endogenous substrates (Marrs, 1996). In maize the GST isozymes have been intensively studied and characterized (Marrs, 1996, and refs. therein; Dixon et al., 1997; Irzyk and Fuerst, 1997). Wheat (*Triticum aestivum* L.) contains GST activity (Jablonkai and Hatzios, 1991; Scarponi et al., 1991; Edwards and Cole, 1996; Riechers et al., 1996b), and several GST isozymes have been identified (Williamson and Beverley, 1988; Mauch and Dudler, 1993). Molecular characterization of wheat GSTs has been limited to the pathogen-induced expression of the wheat *GstA1* gene and its encoded GST 29 protein (Dudler et al., 1991; Mauch and Dudler, 1993). GST 29 has activity with a model, nonherbicidal GST substrate, CDNB (Habig et al., 1974), but does not have activity with the herbicides metolachlor and atrazine (Mauch and Dudler, 1993).

Herbicide safeners are used to protect several monocot crop species from herbicide injury (Hatzios, 1989; Kreuz, 1993), usually by increasing the activity of specific herbicide-metabolizing enzymes (GSTs, Cyt P450-dependent monooxygenases, glucosyltransferases) and therefore the rate of herbicide metabolism (Cole, 1994; Farago et al., 1994; Kreuz et al., 1996). Safeners have also been shown to increase the activity of a vacuolar transporter that pumps glutathione *S*-herbicide conjugates into the vacuole of plant cells (Gaillard et al., 1994).

Wheat can be protected from injury from the chloroacetamide herbicide dimethenamid through the use of herbicide safeners (Riechers et al., 1994, 1996b). We have previously reported that the metabolism of dimethenamid is enhanced in *T. aestivum* shoots grown from safener-treated seed, and that metabolism occurs exclusively through the GSH conjugation system (Riechers et al., 1996a). The objectives of this study were to examine the effects of safener treatment on total GST activity in *T. aestivum* shoots and on individual GST isozymes in *T. aestivum*, *Triticum durum*, and *Triticum tauschii* shoots, and to purify and partially

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; FPLC, fast-protein liquid chromatography; GST, glutathione *S*-transferase; GST-C, glutathione *S*-transferase activity assayed with 1-chloro-2,4-dinitrobenzene as a substrate; GST-D, glutathione *S*-transferase activity assayed with dimethenamid as a substrate.

characterize a safener-induced GST isozyme that uses dimethenamid as a substrate.

Here we report on the partial characterization of GST isozymes in unsafened and safened *T. aestivum*, *T. durum*, and *T. tauschii* shoots, and the purification of a safener-induced GST isozyme (GST TSI-1) from *T. tauschii*, a diploid wheat species considered to be the donor of the D genome found in hexaploid wheat (*T. aestivum*), which contains the A, B, and D genomes (Miller, 1987). Peptide fragments derived from GST TSI-1 share significant amino acid sequence homology with the safener-inducible maize GST V (Irzyk and Fuerst, 1997), two auxin-regulated tobacco (*Nicotiana tabacum*) GSTs, and other type III plant GSTs (Droog et al., 1993, 1995; Marrs, 1996).

MATERIALS AND METHODS

Chemicals

Radiolabeled dimethenamid (3-[^{14}C]thiophene ring, specific activity 50.6 mCi/mmol; for complete chemical name and structure, see Riechers et al., 1996a) and analytical-grade dimethenamid (99.4% pure as determined by GC) were provided by Sandoz Agro, Inc. (Des Plaines, IL). The commercial formulation of fluxofenim (CGA-133205 or Concep III, 959 g of active ingredient/L) and cloquintocet-mexyl (CGA-185072, formulated as 25% active ingredient in a wettable powder) were provided by the Ciba Corp. (Greensboro, NC, and Basel, Switzerland). All other chemicals were purchased from Sigma.

Plant Material

Hexaploid wheat (*Triticum aestivum* L. cv Madsen) seeds were treated with the safener fluxofenim (0.25 g/kg of seed), and seedlings were grown and harvested as previously described (Riechers et al., 1996a). Diploid wheat (*Triticum tauschii* [Coss.] Schmal.) and tetraploid wheat (*Triticum durum* Desf. cv Reva) seeds were treated with the wheat safener cloquintocet-mexyl (Kreuz et al., 1991; Kreuz, 1993) using the soil-drench technique. Seeds of *T. tauschii* had to be removed from the outer husks. Seeds were planted 3 cm deep in plastic pots containing vermiculite. The pots were watered to saturation with 50 μM of the safener cloquintocet-mexyl, covered with aluminum foil, and subjected to prechilling at 4°C for 7 d to increase seed germination.

For analytical anion-exchange FPLC experiments for GST isozyme separation and characterization, pots were removed from prechilling, watered with 50 μM cloquintocet-mexyl, covered with aluminum foil, and incubated at 21°C for 4 d. Etiolated shoots (2–4 cm) were excised directly above the seeds, frozen, and stored in liquid nitrogen until protein extraction. To maximize enzyme yield for GST isozyme purification from *T. tauschii*, plants were grown as described above, but were incubated at 21°C for 8 d and watered with 50 μM cloquintocet-mexyl 1, 4, and 7 d after removal from prechilling. Etiolated shoots (8–12 cm) were excised directly above the seeds, frozen, and stored in liquid nitrogen until protein extraction.

Total GST-D Activity Assay

All tissue homogenization and extraction steps were carried out at 4°C. Crude protein extracts were prepared by homogenizing 0.5 to 1 g of frozen 4-d-old shoots of *T. aestivum* in 5 volumes of extraction buffer containing 250 mM Tris-HCl, pH 7.8, 1 mM Na_2EDTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 50 mg/mL polyvinylpolypyrrolidone in a glass mortar and pestle. The homogenate was then centrifuged at 12,000*g* for 5 min. The supernatant was decanted and used as the source of enzyme for GST-D assays, which were conducted as previously described (Riechers et al., 1996b), except that the reactions were incubated for 30 min instead of 60 min. GST-D activities were corrected for nonenzymatic conjugation of dimethenamid with GSH. The experiment evaluating the influence of fluxofenim on total GST-D activity was conducted twice, with two replications per experiment, and the means from the experiments are reported ± SE.

GSH Assay

GSH levels were determined as previously described (Lamoureux and Rusness, 1986; Viger et al., 1991) with the modifications described below (Davies et al., 1984; Roberts and Francetic, 1993). Crude extracts were prepared by homogenizing 0.5 to 1 g of frozen 4-d-old shoots of *T. aestivum* in five volumes of extraction buffer (0.2% [v/v] trifluoroacetic acid, 0.5 mM Na_2EDTA) in a glass mortar and pestle. The homogenate was then centrifuged at 12,000*g* for 5 min, the supernatant was decanted, and the volume was recorded. GSH levels were determined immediately after obtaining crude extracts by measuring the conjugation of GSH with excess CDNB. The reaction mixture consisted of 150 mM potassium phosphate buffer, pH 6.5, 1 mM CDNB, 1.5 units of equine GST (Sigma), and 200 μL of crude extract or GSH standards (in extraction buffer) in a total volume of 3 mL. The reaction was incubated at 21°C for 12 min, and initial and final A_{340} values were recorded.

The overall change in A_{340} was used to determine GSH levels by comparison with a GSH standard curve. Values reported are corrected for GSH oxidation that occurred during sample preparation and analysis by measuring oxidation of a GSH-spiked tissue sample that was extracted and assayed concurrently with the above procedure. The experiment evaluating the influence of fluxofenim on GSH levels was conducted twice, with two replications per experiment, and the means from the experiments are reported ± SE.

Analytical Anion-Exchange FPLC Separation of GST Isozymes in Safened and Unsafened *T. aestivum*, *T. durum*, and *T. tauschii*

Crude protein extracts were prepared by homogenizing 5 to 10 g of frozen 4-d-old *T. aestivum*, *T. tauschii*, and *T. durum* shoots in 5 volumes of extraction buffer in a mortar and pestle. The crude homogenate was filtered through eight layers of cheesecloth and centrifuged at 20,000*g* for 15 min. The supernatant was decanted and adjusted to 40%

saturation with solid ammonium sulfate, stirred for 15 min, and centrifuged at 20,000*g* for 20 min. The supernatant was decanted and adjusted to 90% saturation with solid ammonium sulfate, stirred for 15 min, and centrifuged at 40,000*g* for 20 min. Approximately 90% of the GST-D activity in the crude extract was precipitated in this fraction. The supernatant was discarded and the pellets were frozen in liquid nitrogen and stored overnight at −80°C.

The pellets were resuspended in TB buffer (20 mM Tris-HCl, pH 7.8, 5 mM β-mercaptoethanol) and desalted into TB buffer using PD-10 columns (Pharmacia). The protein solution was then filtered through a 0.45-μm membrane syringe filter (Nalgene, Rochester, NY), and 10 mg of total protein was loaded at 0.5 mL/min onto an analytical Mono-Q anion-exchange column (Pharmacia HR 5/5) equilibrated in TB buffer. FPLC was conducted as described previously (Fuerst et al., 1993), except that the following NaCl gradient in TB buffer was used: 0 to 50 mM NaCl in 1 mL, 50 to 180 mM NaCl in 40 mL, and 180 to 500 mM NaCl in 14 mL. Fractions (1 mL) were collected and assayed for GST-D activity as described above, and for GST-C activity as previously described (Irzyk and Fuerst, 1993). Anion-exchange FPLC was conducted at least twice for both unsafened and safened *Triticum* spp., and representative results from one experiment are shown.

Enzyme Purification and Amino Acid Sequencing

Cloquintocet-mexyl-treated *T. tauschii* shoots (28 g) were pulverized in a mortar and pestle with 5 volumes of extraction buffer as described above. Crude extract preparation, ammonium sulfate fractionation, and desalting procedures were identical to those described above for analytical anion-exchange FPLC. The desalted 40 to 90% ammonium sulfate fraction (about 83 mg of protein) was filtered through a 0.45-μm membrane syringe filter (Nalgene) and loaded at 5 mL/min onto a preparative (6-mL column volume) anion-exchange FPLC column (Resource Q, Pharmacia) equilibrated in TB buffer. The following NaCl gradient was used to elute GSTs from the column: 0 to 50 mM NaCl in 10 mL, 50 to 180 mM NaCl in 240 mL, and 180 to 500 mM NaCl in 90 mL. Fractions (5 mL) were collected and assayed for GST-D and GST-C activity as described above.

The three 5-mL fractions containing the highest GST-D activity (fractions 22–24) were pooled and desalted on PD-10 columns into TB buffer. The protein solution (about 8 mg) was filtered and subjected to analytical anion-exchange Mono-Q FPLC as described above. The seven 1-mL fractions containing the highest GST-D activity were pooled and loaded at 0.3 mL/min directly onto a dimethenamid-glutathione-affinity column (synthesis protocol described below) previously equilibrated in TB buffer. The column was washed sequentially with 5 mL of TB buffer, 5 mL of TB buffer plus 250 mM NaCl, and 5 mL of TB buffer, and was then eluted with 5 mL of 60 mM Tris-HCl, pH 9.0, plus 20 mM GSH. The column flow-through was reloaded onto the column and the washing and elution procedures repeated. The column eluates (10 mL) were pooled, desalted into TB buffer, and concentrated to about 1 mL with a hollow fiber concentrator/filtrator (Irzyk and Fuerst, 1993).

The dimethenamid-glutathione-affinity column was constructed using a modification of a protocol provided by K. Kreuz (Novartis Corp., Basel, Switzerland, personal communication). The GSH conjugate of dimethenamid was synthesized and purified as previously described (Riechers et al., 1996a) and dried by vacuum centrifugation (Speed-Vac, Savant, Farmingdale, NY). About 50 μmol of dimethenamid-GSH conjugate was dissolved in 1 M K_2HPO_4, pH 8.2, and added to 0.2 g of hydrated (swells to 1 mL) vinyl sulfone-activated agarose (Sigma; 11 μmol active vinyl groups/mL, hydrated and equilibrated in 0.2 M K_2HPO_4, pH 8.2). The conjugate was incubated with the activated agarose at 21°C and gently stirred for 2 d. The agarose was then blocked with 50 mg/mL Gly, pH 9, overnight to ensure that all vinyl groups had been derivatized. The dimethenamid-GSH linked agarose was then poured into a 3-mL syringe barrel and equilibrated with 20 mM Tris-HCl, pH 7.8.

The final protein purification step involved the use of preparative two-dimensional PAGE to resolve the proteins that bound to and eluted from the affinity column. The affinity column eluate was concentrated to 0.1 mL by vacuum centrifugation. One-half of this sample was subjected to preparative two-dimensional PAGE using the protocol described below. The gels were stained with Coomassie blue R-250 and destained, and protein spots were excised with a cork borer. Gel slices were washed with water to remove methanol and acetic acid, lyophilized, and then rehydrated in 100 mM Tris-HCl, pH 7.8, 0.05% SDS, and digested in gel with 3 pmol of endoproteinase Lys-C at 37°C for 18 h to generate peptide fragments. Peptide fragments were resolved by reverse-phase HPLC on a narrow-bore C_{18} column (Vydac, Hesperia, CA) using a linear 3.5 to 31.5% acetonitrile (containing 0.1% trifluoroacetic acid) gradient in 36 min at a flow rate of 0.2 mL/min. Peptide fragment elution was monitored at A_{210}. Selected peptides were subjected to amino acid sequencing using Edman chemistry on a liquid-pulse protein sequenator (Procise, Applied Biosystems). Protein digestions, HPLC, and amino acid sequencing were performed by the Protein and Nucleic Acid Facility, Beckman Center, Stanford University Medical Center (Stanford, CA).

Electrophoresis and Immunoblot Analysis

One-dimensional SDS-PAGE was conducted according to the method of Laemmli (1970) using 17.5% resolving gels (30:0.174 acrylamide:piperazine diacrylamide). Two-dimensional PAGE was conducted according to the method of O'Farrell (1975). First-dimension IEF was performed using tube gels containing ampholytes of pH 6.0 to 8.0 (Bio-Rad) and pH 3.0 to 10.0 (Pharmacia) blended 4:1 (v/v), respectively. Preparative IEF was performed for 7400 V × h with 3-mm tube gels using a vertical gel electrophoresis system (BRL), and analytical IEF was performed for 4000 V × h with 1-mm tube gels using a miniformat system (Bio-Rad). Second-dimension SDS-PAGE was conducted using 17.5% acrylamide gels (3-mm

Table I. *Total GST-D activities and GSH levels in unsafened and safened hexaploid wheat (T. aestivum) shoots*

GST-D activity or GSH were extracted from 4-d-old *T. aestivum* shoots as described in "Materials and Methods." Safened *T. aestivum* was treated with a seed application of fluxofenim at 0.25 g/kg of seed.

Treatment	Total GST-D activity	GSH
	pmol min^{-1} mg^{-1} protein	*μmol g^{-1} fresh weight*
Unsafened	75 (9)[a]	4.4 (0.2)
Safener-treated	693 (39)	4.1 (0.4)

[a] Numbers in parentheses indicate SE.

thick for preparative, 1-mm thick for analytical) as described above. Proteins were visualized by either Coomassie blue R-250 or silver-staining (Rabilloud et al., 1988).

For immunoblotting experiments, gels were equilibrated in transfer buffer (48 mM Tris base, 39 mM Gly, 0.0375% SDS, 20% methanol) for 30 min, then electrophoretically transferred to 0.45-μm nitrocellulose membranes (Hybond-C Extra, Amersham) using a semidry transfer apparatus (Trans-Blot SD, Bio-Rad) operating at 15 V for 50 min. Membranes were then incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% [w/v] nonfat dry milk, 0.02% sodium azide) at least 3 h before being probed overnight with a primary antiserum raised against a mixture of maize (*Zea mays*) GSTs (provided by K. Kreuz, Novartis Corp.) diluted 1:1000 in blocking buffer. The antiserum was raised against a mixture of maize GST subunits with molecular masses of 26 and 27 kD, which had been purified from benoxacor-induced etiolated maize shoots by anion-exchange chromatography on Q-Sepharose and *S*-hexylglutathione-affinity chromatography (K. Kreuz, personal communication). Blots were washed with blocking buffer, then incubated overnight with secondary antibody (anti-rabbit IgG conjugate to alkaline phosphatase, Sigma) at a dilution of 1:4000 in blocking buffer. Blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as alkaline phosphatase substrates (Harlow and Lane, 1988).

Protein Determination

Protein was quantified using the method of Bradford (1976) with BSA as a standard, by A_{280}, or by visual estimation using SDS-PAGE and Coomassie or silver-staining in comparison with BSA standards.

RESULTS AND DISCUSSION

Total GST-D Activity and GSH Levels in Safened and Unsafened *T. aestivum*

T. aestivum has very low, constitutively expressed GST-D activity (Table I) (Riechers et al., 1996b), which may account for the susceptibility of *T. aestivum* to the herbicide dimethenamid (Riechers et al., 1994). Seed treatment with the herbicide safener fluxofenim increased total GST-D activity 9-fold in *T. aestivum* shoots (Table I). This increase in total GST-D activity correlates well with the increase in dimethenamid metabolism in *T. aestivum* shoots observed in response to fluxofenim (Riechers et al., 1996a). Fluxofenim treatment had no effect on GSH levels in *T. aestivum* shoots (Table I). In comparison, safeners increased GST activity, using chloroacetamide herbicide substrates in maize (Viger et al., 1991; Fuerst and Lamoureux, 1992) and grain sorghum (*Sorghum bicolor*) (Gronwald et al., 1987), but had little or no effect on GSH levels. Therefore, safener response is generally attributed to increased GST activity (Gronwald et al., 1987; Komives and Dutka, 1989; Viger et al., 1991; Fuerst and Lamoureux, 1992; Farago et al., 1994). Our results indicate that increased GST-D activity, and not GSH, is the primary basis for the protection of *T. aestivum* from the herbicidal effects of dimethenamid.

Analytical Anion-Exchange FPLC Separation of GST Isozymes in Safened and Unsafened *T. aestivum*, *T. durum*, and *T. tauschii*

Anion-exchange FPLC was used to separate total GST activity in crude extracts from *T. aestivum* shoots into component activities, as has been reported previously for maize (Fuerst et al., 1993) and sorghum (Dean et al., 1990). Three activities were resolved in unsafened *T. aestivum* that exhibited GST-C activity, including one major constitutive GST-C activity that eluted first from the column (Fig. 1A). There was very little GST-D activity in unsafened *T. aestivum* (Fig. 1A) relative to fluxofenim-treated *T. aestivum* (Fig. 1B). Fluxofenim-treated *T. aestivum* contained six re-

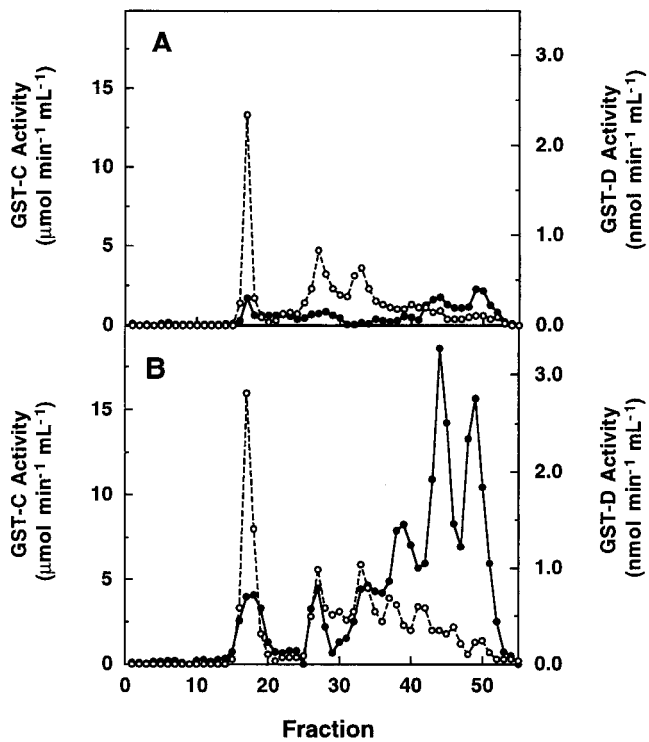

Figure 1. Analytical anion-exchange FPLC separation of GST activities present in unsafened (A) or fluxofenim-safened (B) hexaploid wheat (*T. aestivum* cv Madsen) shoots. Fractions (1 mL) were collected and assayed for GST-C activity (○) and GST-D activity (●).

solved activities that used dimethenamid as a substrate, including three major GST-D activities (fractions 37–52), all of which are induced by the safener fluxofenim (Fig. 1B). In contrast, there was very little induction of the constitutively expressed GST-C activities (Fig. 1B). The greater level of safener induction of GST activities that use chloroacetamide herbicide substrates than that of GST-C activities was also reported in maize (Fuerst et al., 1993) and sorghum (Dean et al., 1990). Treatment of *T. aestivum* with the safener cloquintocet-mexyl as a seed treatment (1.25 g/kg of seed) resulted in almost identical FPLC profiles as fluxofenim treatment (qualitatively), so only fluxofenim-treated *T. aestivum* is shown (Fig. 1B). Results of the anion-exchange FPLC experiments are consistent with those shown in Table I, in which total GST-D activity was low in unsafened *T. aestivum* shoots and was induced 9-fold by fluxofenim treatment.

Anion-exchange FPLC was also used to separate total GST activity in crude extracts from *T. tauschii* and *T. durum* shoots into component activities (Figs. 2 and 3). *T. tauschii* was qualitatively similar to *T. aestivum* with respect to the major GST-D activities resolved in both unsafened and safened shoots, although the activity levels were lower in cloquintocet-mexyl-treated *T. tauschii* (Fig. 2B) compared with fluxofenim-treated *T. aestivum* (Fig. 1B). We have previously shown that GST-D activities are very low in crude extracts prepared from unsafened *T. aestivum*, *T.*

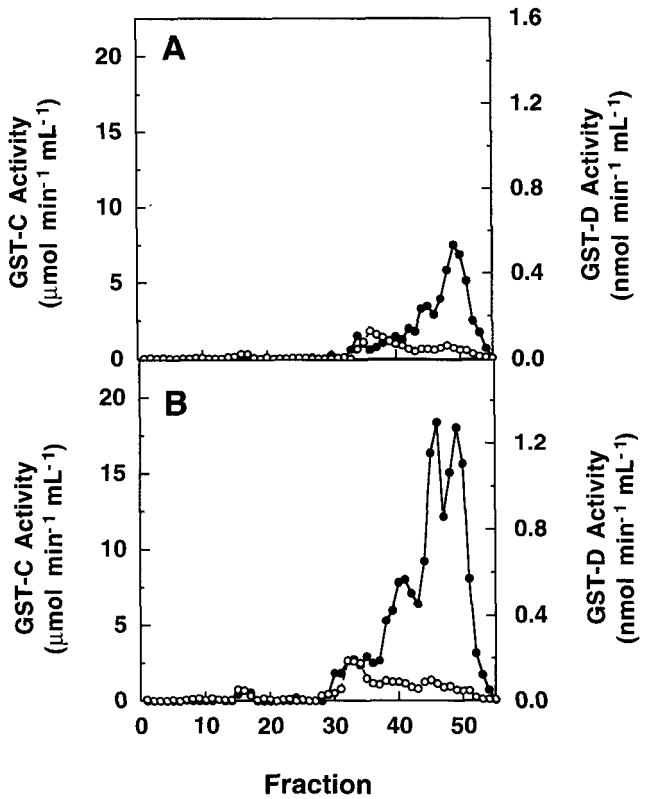

Figure 2. Analytical anion-exchange FPLC separation of GST activities present in unsafened (A) or cloquintocet-mexyl-safened (B) diploid wheat (*T. tauschii*) shoots. Fractions (1 mL) were collected and assayed for GST-C activity (○) and GST-D activity (●).

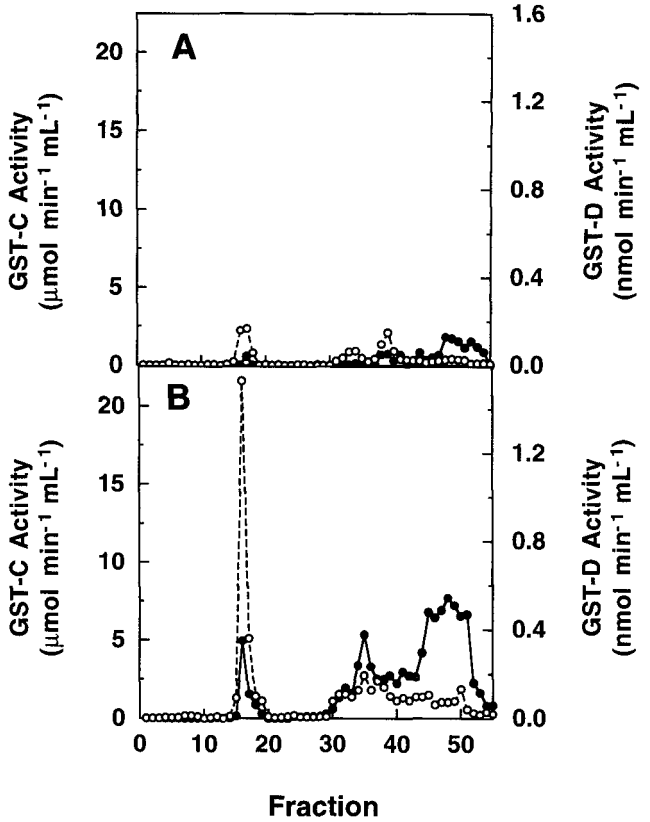

Figure 3. Analytical anion-exchange FPLC separation of GST activities present in unsafened (A) or cloquintocet-mexyl-safened (B) tetraploid wheat (*T. durum* cv Reva) shoots. Fractions (1 mL) were collected and assayed for GST-C activity (○) and GST-D activity (●).

tauschii, and *T. durum*, and that fluxofenim is a better inducer of GST-D activity than cloquintocet-mexyl in wheat and wheat relatives (Riechers et al., 1996b). One major difference noted was that the predominant GST-C activity in *T. aestivum* that eluted from the column first was present at a much lower level in both unsafened and safened *T. tauschii* (Fig. 2). This may be due to the difference in genetic composition between the two species. This predominant GST-C activity was present in both unsafened and cloquintocet-mexyl-treated *T. durum*, which is a tetraploid wheat species that contains the A and B genomes (Fig. 3).

In contrast to *T. aestivum* and *T. tauschii*, the major GST-D activities were present at much lower levels in cloquintocet-mexyl-treated *T. durum* (Fig. 3B). We have previously suggested that the D genome may be an important source of GST isozymes that metabolize dimethenamid and are involved in the safener response in hexaploid and diploid wheat (Riechers et al., 1996b). Edwards and Cole (1996) also noted that tetraploid wheat species with the AABB genome designations did not respond as well as hexaploid wheat to the safener fenchlorazole-ethyl when assaying for GST activity with the herbicides fluorodifen and fenoxaprop. The GST gene(s) in *T. aestivum* and *T. durum* that encodes the isozyme with the predominant GST-C activity, however, appears to be present in the A and/or B genome(s).

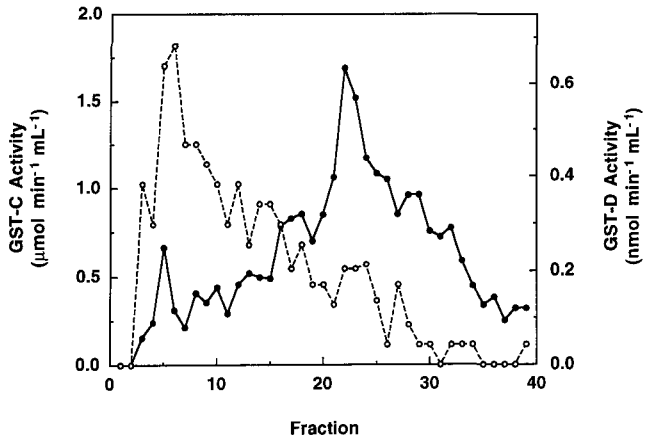

Figure 4. Preparative anion-exchange FPLC separation of GST activities present in cloquintocet-mexyl-treated *T. tauschii* shoots. Fractions (5 mL) were collected and assayed for GST-C activity (○) and GST-D activity (●). Fractions 22 to 24 were pooled and subjected to further purification steps as outlined in "Materials and Methods."

Enzyme Purification

T. tauschii responds to safeners in a manner similar to *T. aestivum* with respect to increased total GST-D activity (Riechers et al., 1996b) and safener-induced GST-D activities resolved by anion-exchange FPLC (Figs. 1 and 2). Diploid wheat lacks the homoeologous chromosome groups found in hexaploid wheat, and theoretically should contain one-third as many GST genes and isozymes, which should simplify protein purification and subsequent molecular cloning and characterization. Therefore, *T. tauschii* was used for GST purification. To generate as much shoot fresh weight as possible from the limited amount of seed available, the safener cloquintocet-mexyl was used because of its lower phytotoxicity to wheat seedlings compared with fluxofenim (Riechers et al., 1996b); the shoots were also allowed to grow for 8 instead of 4 d. Protein purification techniques were used that were similar to those used previously to purify the maize safener-inducible GST IV (Irzyk and Fuerst, 1993), including anion-exchange FPLC and affinity chromatography. However, to bind only the GSTs that could use dimethenamid as a substrate, a dimethenamid-affinity column was constructed and utilized instead of an *S*-hexylglutathione-affinity column.

The preparative anion-exchange FPLC separation of GST activities in cloquintocet-mexyl-treated *T. tauschii* shoots is shown in Figure 4. The chromatographic separation was similar to that shown in Figure 2B (cloquintocet-mexyl-treated *T. tauschii*) in that the predominant GST-C activities eluted from the column first, and the predominant GST-D activities eluted later in the gradient; however, the resolution was reduced. The differences in resolution and retention time are most likely due to the use of a preparative Resource Q column for enzyme purification (Fig. 4), as opposed to the analytical Mono-Q column separation shown in Figure 2B.

The highest GST-D activity was bordered by two lower GST-D activities in all three chromatographic separations of GSTs from safened *T. aestivum* and *T. tauschii* shoots (Figs. 1B, 2B, and 4). The highest GST-D activity from cloquintocet-mexyl-treated *T. tauschii* (Fig. 4, fractions 22–24) also showed the highest level of safener induction (Fig. 2); this activity was chosen for further enzyme purification by Mono-Q FPLC and dimethenamid-affinity chromatography. Since only one GST-D activity was chosen for further purification, there was a large decrease in yield observed during the Resource Q FPLC step (Table II). This was due to the elimination of all but one of the GST-D activities, as well as choosing only the fractions with the highest GST-D activity. Mono-Q FPLC provided further protein purification of the major 26-kD band (Fig. 5, lane 5), as also noted by the corresponding increase in specific activity (Table II), and concentrated the protein for dimethenamid-affinity chromatography. Only one major GST-D activity was present in the Mono-Q FPLC profile (data not shown), which verifies that two GST-D activities had not merged in the Resource Q FPLC separation (Fig. 4).

Dimethenamid-affinity chromatography was the most effective purification step, resulting in a more than 49-fold increase in specific activity relative to the previous step, and a 220-fold increase in specific activity relative to the crude extract (Table II). Analysis of purification steps by SDS-PAGE showed that the major band at about 26 kD in the Mono-Q fraction (Fig. 5, lane 5) had reduced intensity in the affinity column flow-through (Fig. 5, lane 6), and was

Table II. *Purification of GST TSI-1 from cloquintocet-mexyl-treated T. tauschii shoots*

The GST TSI-I enzyme was extracted from 28 g of etiolated, 8-d-old *T. tauschii* shoots treated with cloquintocet-mexyl applied as a soil drench. GST-D activity was determined with radiolabeled dimethenamid as a substrate as described in "Materials and Methods."

Purification Step	Total Protein	Total Activity	Specific Activity	Yield[a]	Purification[a]
	mg	*units*[b]	*units mg*$^{-1}$	*%*	*-fold*
Crude extract	132	23.5	0.18	100	1
Ammonium sulfate	83	21.0	0.25	89.4	1.4
Resource Q FPLC	8.4	4.6	0.55	19.6	3.1
Mono-Q FPLC	3.2	2.6	0.81	11.1	4.5
Dimethenamid-affinity chromatography	0.05	2.0	40	8.5	222

[a] Yield and purification values are based on total GST-D activity in the crude extract. [b] One unit is defined as 1 nmol of dimethenamid conjugated per min.

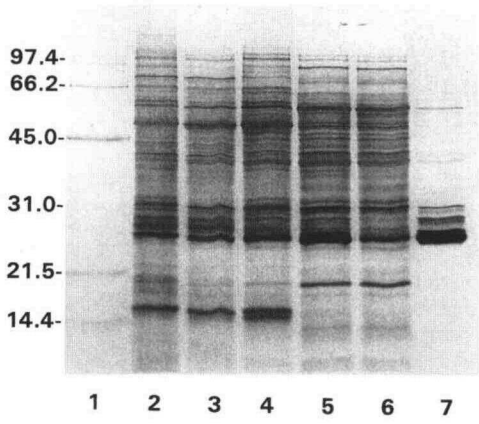

Figure 5. SDS-PAGE analysis of GST purification from cloquintocet-mexyl-treated *T. tauschii* shoots. Proteins were visualized by silver-staining. Lane 1, Molecular mass standards (kD); lane 2, crude extract; lane 3, proteins precipitated between 40 and 90% ammonium sulfate; lane 4, Resource Q anion-exchange FPLC fractions containing the highest GST-D activities; lane 5, Mono-Q anion-exchange FPLC fractions containing the highest GST-D activities; lane 6, dimethenamid-affinity column flow-through (twice through the column); lane 7, dimethenamid-affinity column eluate. Approximately 3.5 μg of protein was loaded in each of lanes 2 to 6.

eluted with GSH from the affinity column (Fig. 5, lane 7). However, the presence of several polypeptides with molecular masses between 25 and 31 kD in the dimethenamid-affinity column eluate suggested that many GST subunits may have been binding to and eluting from the column (Fig. 5, lane 7).

Since several GST subunits may have been present in the affinity column eluate (Fig. 5, lane 7), two-dimensional PAGE was used to isolate the major GST subunit present (Fig. 6). There appeared to be three proteins with very similar pI values and molecular masses comprising the major band at 26 kD on SDS-PAGE (Fig. 5). These three proteins (Fig. 6, A, B, and C) were excised separately and digested with endoproteinase Lys-C to generate peptide fragments. Reverse-phase HPLC separation of these fragments generated peptide maps for each of the three proteins (data not shown). Based on the similarity of the peptide maps, the three proteins may be charge isomers of the same protein, posttranslationally modified isoforms of the same protein, or very similar but distinct proteins. Selected fragments were chosen from the most abundant protein (Fig. 6, protein B) for amino acid sequence analysis, and several corresponding fragments were chosen from proteins A and C for comparison with protein B.

Immunoblot Analysis

A maize GST antiserum has been shown to detect several GST isozymes in *T. aestivum* (Mauch and Dudler, 1993; Riechers et al., 1996b) and soybean (*Glycine max*) (Flury et al., 1995, 1996). The major 26-kD polypeptide that was enriched in all purification fractions (Fig. 5), as well as several minor proteins in the molecular mass range of 26 to 31 kD, were detected by the antiserum (Fig. 7). The decrease in the quantity of the 26-kD polypeptide(s) when comparing lanes 5 (before affinity column) and 6 (column flow-through) (Fig. 5) correlates well with the decrease in band intensity observed in the immunoblot in lane 4 versus lane 5, respectively (Fig. 7). This provides evidence that the GST subunits bind to the affinity column. An immunoblot of a two-dimensional PAGE separation of the proteins that eluted from the dimethenamid-affinity column (Fig. 5, lane 7) showed that proteins A, B, and C, as well as other minor proteins, were detected by the antiserum (data not shown). This result provides further evidence that GSTs were binding to and eluting from the dimethenamid-affinity column.

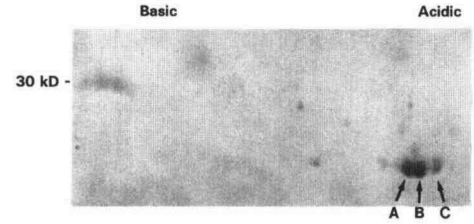

Figure 6. Two-dimensional PAGE separation of proteins bound to and eluted from the dimethenamid-affinity column (Fig. 5, lane 7). Proteins were visualized by Coomassie blue R-250 staining. Proteins A, B, and C were excised from the gel and separately subjected to protease digestion, and peptide fragments were resolved by reverse-phase HPLC. Selected peptide fragments were analyzed for amino acid sequence.

Amino Acid Sequences

Three peptide fragments from protein B (Fig. 6) yielded amino acid sequences. Those obtained from proteins A and C (Fig. 6) were identical to those obtained from corresponding fragments from protein B (data not shown). Protein B was renamed GST TSI-1 (T for *Triticum* and SI for safener-induced). The sequences from GST TSI-1 purified from *T. tauschii* were compared with other published plant GST sequences. GST TSI-1 had the highest homology with sequences from the safener-inducible maize GST V (Irzyk and Fuerst, 1997), and also had homology to two auxin-regulated tobacco GSTs (Droog et al., 1993) (Table III), suggesting that GST TSI-1 may be a type III plant GST according to the classification system proposed by Droog et al. (1995). One of the amino acids in GST TSI-1 peptide 1, Arg-17 (relative to maize GST III numbering, Table III), is

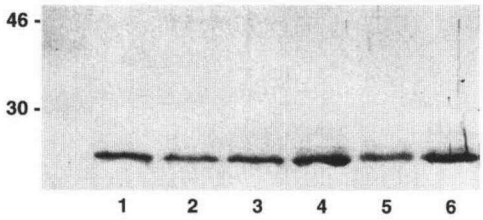

Figure 7. Immunoblot of *T. tauschii* proteins from the GST purification steps. The gel was identical to the one shown in Figure 5. The blot was probed with a maize GST antiserum and developed as described in "Materials and Methods." Lanes 1 to 6 correspond to lanes 2 to 7, respectively, in Figure 5.

Table III. *Partial amino acid sequences of various plant GSTs and GST TSI-1 from T. tauschii*

The sequences for GST TSI-1 were obtained from peptides generated by endoproteinase Lys-C digestion of two-dimensional PAGE-purified enzyme. Asterisks (*) indicate identity with maize GST V. Unidentified amino acids are denoted with an X.

Species	Amino Acids	Sequence
Maize GST V	11–24	L L D F W V S P F G Q R C R
Maize GST II/IV[a]	8–21	V Y G W A I * * * V S * A L
Maize GST III	6–19	* Y G M P L * * N V V * V A
Maize GST I	6–19	* Y G A V M * W N L T * * A
Wheat GST 29	6–19	V F G H P M L T N V A * V L
Wheat GST TSI-1 (peptide 1)		* * G A X P X * * V T * V K
Tobacco NT 103	6–19	* * G * * Y * * * T H * V E
Tobacco NT 107	8–21	* * * * * P * M * * M * L *
Maize GST V	31–43	G L A Y E Y L E Q D L G K
Maize GST II/IV	28–40	* V D * * L V P M S R Q D
Maize GST III	26–38	* * D F * I V P V * * T T
Maize GST I	26–38	* S D * * I V P I N F A T
Wheat GST 29	26–38	* A E * * L V P M * F V A
Wheat GST TSI-1 (peptide 2)		* * S * * D V * E * * Y *
Tobacco NT 103	26–38	* V K * * * I * E * R D N
Tobacco NT 107	28–40	E I K * * * K * E * * R N
Maize GST V	180–193	S A E K E C P R L A T W A K
Maize GST II/IV	187–200	H * L P H V S A W W Q G L A
Maize GST III	187–200	A * R P H V K A W W E A I A
Maize GST I	185–198	D * Y P H V K A W W S G L M
Wheat GST 29	194–207	D D Y P K V K A W W E M L M
Wheat GST TSI-1 (peptide 3)		A * L P D V G * * L E F * *
Tobacco NT 103	173–186	V T S E K F * N F S R * R D
Tobacco NT 107	176–189	* T * A * * * K F V A * * *

[a] Maize GSTs II and IV share a common 27-kD subunit (Jepson et al., 1994; Irzyk et al., 1995).

highly conserved among plant and animal GSTs, and may be involved in the binding and stabilization of the glutathione anion (Droog et al., 1993). Limited homology was also observed among GST TSI-1 and other plant GSTs from wheat (GST 29) and maize (GST I, II/IV, and III) (Grove et al., 1988; Dudler et al., 1991; Jepson et al., 1994; Irzyk et al., 1995).

It is possible that GST TSI-1 is the same as the 26-kD GST (designated GST 26) that was induced by herbicide and cadmium treatment in *T. aestivum* (Mauch and Dudler, 1993), because both were recognized by the same maize GST antiserum (Fig. 7) (Mauch and Dudler, 1993). If this is true, then the observation that GST 29 is immunologically unrelated to GST 26 and has different inducibility and enzymic properties (Mauch and Dudler, 1993) is supported by the finding that, based on partial amino acid sequence analysis, GST TSI-1 shares less sequence identity with the *T. aestivum* GST 29 than with the maize GST V (Table III).

SUMMARY

Herbicide safeners induce specific GSTs in wheat that can metabolize the chloroacetamide herbicide dimethenamid. GST isozymes in *T. aestivum* and *T. tauschii* that have the highest activity with dimethenamid as a substrate are induced by safeners to a much greater extent than GST isozymes that have the highest activity with CDNB as a substrate (Figs. 1 and 2), and appear to be encoded by GST genes in the D genome of hexaploid wheat (ABD genomes) or diploid wheat (D genome only). In contrast, the major GST-D activities in the tetraploid wheat *T. durum* (AB genomes) were not induced by safener to the same extent as in *T. aestivum* or *T. tauschii*.

A safener-induced GST from *T. tauschii* with a subunit molecular mass of 26 kD was purified more than 220-fold using ammonium sulfate fractionation, anion-exchange FPLC, and dimethenamid-affinity chromatography; this protein was then further purified by two-dimensional PAGE. Although the purified protein was not directly shown to have activity with dimethenamid as a substrate, there is evidence that it metabolizes dimethenamid, including the fact that the 26-kD GST subunit bound to a dimethenamid-affinity matrix, and that this subunit comprised the greatest proportion of the polypeptides that bound to and eluted from the dimethenamid-affinity column. This fraction had a more than 220-fold increase in specific activity relative to the crude extract. Amino acid sequence comparison with other plant GSTs indicates that the *T. tauschii* GST TSI-1 may be most similar to the auxin-regulated type III plant GSTs (Droog et al., 1993, 1995; Marrs, 1996; Andrews et al., 1997); however, analysis of a full-length cDNA or genomic sequence is needed to conclusively determine this relationship (see "Note Added in Proof").

GST TSI-1 appears to be different from the only other wheat GST that has been characterized at the molecular level, GST 29 (Mauch and Dudler, 1993). In addition to the low amino acid sequence identity when comparing GST 29

with GST TSI-1 (Table III), expression of GST 29 (protein and mRNA transcripts) was induced by pathogen attack but not by herbicides, and the enzyme did not use the chloroacetamide herbicide metolachlor as a substrate. It was speculated that GST 29 was specifically involved in defense against pathogen attack and not in xenobiotic metabolism (Mauch and Dudler, 1993). In contrast, GST TSI-1 appears to belong to a different class of wheat GSTs that is regulated by xenobiotics and involved in xenobiotic metabolism.

NOTE ADDED IN PROOF

A full-length cDNA (accession no. AF004358) has been obtained from *T. tauschii* that contains an open reading frame encoding the partial amino acid sequences of GST TSI-1 reported in this paper, and a Plant Gene Register article discussing this cDNA has been accepted for publication in *Plant Physiology* (PGR 97–110). We would also like to point out that the partial amino acid sequence of tobacco GST NT103 (amino acids 26–38) reported in this paper is correct, according to the sequence reported in GenBank for pCNT103 (accession no. X56263). This same partial sequence reported in Droog et al. (1993) and Jepson et al. (1994) is incorrect.

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