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ORIGINAL ARTICLE

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Tissue-specific expression and localization of safener-induced glutathione *S*-transferase proteins in *Triticum tauschii*

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Abstract Glutathione *S*-transferase (GST; EC 2.5.1.18) gene expression was examined in the coleoptile and new leaf tissue of etiolated shoots of the diploid wheat species *Triticum tauschii* (Coss.) Schmal., which is considered to be a progenitor and the D-genome donor to cultivated, hexaploid bread wheat *Triticum aestivum* L. GST expression (mRNA, protein, and enzyme activity with a herbicide substrate) in these shoot tissues was examined in response to herbicide safener treatment. Two different antibody probes, raised against the same safener-inducible GST protein (*Tt*GSTU1) but differing in their specificity, were utilized to determine tissue distribution and subcellular localization of GST proteins in etiolated shoots. GST transcripts, immunoreactive GST proteins, and herbicide-metabolizing activity were all highest in the coleoptile of etiolated, safener-treated *T. tauschii* shoots. Anti-GST immunolabeling was strongest in the outer epidermal and adjoining sub-epidermal cells in both coleoptiles and new leaves following safener treatment. Our data indicate that safeners protect grass crops from herbicide injury by dramatically inducing the expression of GST proteins in the outer cell layers of the coleoptile, which prevents the herbicide from reaching the sensitive new leaves of etiolated shoots as they emerge from the soil.

Keywords Coleoptile · Detoxification · Gene expression · Herbicide safeners · Protein localization · *Triticum*

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Abbreviation GST: glutathione *S*-transferase

Introduction

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a family of multifunctional enzymes that are common to all plants (Edwards et al. 2000; McGonigle et al. 2000; Wagner et al. 2002). GST protein families are composed of multiple isozymes (as either hetero- or homodimers) that differ in their expression patterns, substrate specificities, and biochemical functions (McGonigle et al. 2000; Dixon et al. 2002). GSTs are expressed in response to numerous endogenous and xenobiotic stresses, and are well known for their involvement in glutathione conjugation of toxic electrophilic molecules (Dixon et al. 2002). GST enzymatic activity may involve direct glutathione conjugation to toxic electrophiles, or glutathione-dependent peroxidase activity, using glutathione as reductant for the detoxification of toxic oxygen species, oxygen radicals, and lipid peroxides formed during or after plant stress (Dixon et al. 1998, 2002; Edwards et al. 2000). Another potential function of plant GSTs is that they may act as carrier proteins for endogenous substrates and secondary compounds by transporting them from the cytosol to the vacuole (Mueller et al. 2000; Walbot et al. 2000). One biochemical function of GST proteins that is well defined is their role in herbicide metabolism in crops. GSTs are the predominant detoxification enzymes in maize and cereal crops that are responsible for metabolism of triazines, acetamide and thiocarbamate herbicides, and certain graminicides such as fenoxaprop-ethyl (Cole 1994; Edwards and Dixon 2000).

Herbicide safeners are chemical compounds that increase the tolerance of certain grass crops (e.g., maize, grain sorghum, wheat, rice) to herbicides (Hatzios and Hoagland 1989; Davies and Caseley 1999). Herbicide safeners protect crop plants by increasing herbicide metabolism and detoxification pathways (Fuerst and Gronwald 1986; Hatzios 1991; Farago et al. 1994; Riechers et al. 1996a; Davies and Caseley 1999). The

increase in metabolism results from enhanced activity of herbicide detoxification enzymes, such as GSTs, cytochrome P450-dependent monooxygenases, and glucosyltransferases (Gronwald et al. 1987; Cole 1994; Kreuz et al. 1996; Brazier et al. 2002). Evidence for coordinate regulation of the entire detoxification pathway by safeners has also been reported. Safeners have been shown to induce the activity of a vacuolar transporter in barley (Gaillard et al. 1994), and the expression of a multidrug resistance-associated protein homologue was induced by the safener cloquintocet-mexyl in combination with phenobarbital in wheat, with the protein being localized to the tonoplast (Theodoulou et al. 2003).

Despite the well-documented induction of GST enzyme activity in grass crops, very little information has been reported on the localization or tissue distribution of the GST enzymes that metabolize herbicides in grass seedlings. Most studies that examined GST enzymatic activity or expression levels (mRNA or protein) in response to safeners have used whole shoots or roots for RNA or protein extraction (Gronwald et al. 1987; Irzyk and Fuerst 1993; Edwards and Cole 1996; Riechers et al. 1996b, 1997, 1998; Cummins et al. 1997; Wu et al. 1999; Brazier et al. 2002; Xu et al. 2002). In safener-treated maize seedlings, it was reported that the majority of metabolism in the shoot (via glutathione conjugation) of the herbicide metazachlor was occurring in the coleoptile (Fuerst et al. 1991; Fuerst and Lamoureux 1992). However, the relative abundance of GST proteins was not examined in the dissected maize tissues used in this study. The subcellular location of GSTs has usually been presumed to be cytosolic. This has been largely based on a lack of protein-targeting sequences detected in their genes and encoded proteins, or differential centrifugation properties of the GST proteins from crude protein extracts, where they are usually reported as being soluble and not membrane-bound (Dixon et al. 2002). However, no direct GST protein localization studies have been conducted in grasses to confirm these preliminary speculations based on sequence analyses or centrifugation properties.

Only a few studies have addressed the distribution of GST proteins in plant tissues other than those in grass shoots or roots. Sari-Gorla et al. (1993) conducted a study utilizing starch gel electrophoresis and isozyme analysis to examine the tissue distribution and developmental regulation of GST proteins in control and herbicide-treated maize inbred lines that differ in their herbicide tolerance. Many different GST isoforms (both constitutive and herbicide-inducible) were found to be regulated and expressed in the various tissues and organs examined, suggesting a complex developmental regulation of GST expression throughout the plant. Holt et al. (1995) used GST subunit-specific antisera and immunoblotting to examine the distribution of specific GST isoforms (subunits) in both untreated and safener-treated maize seedlings and mature plants. The maize GST-29 (*Zm*GSTF1) subunit was expressed constitutively throughout the plant, while the maize GST-27 (*Zm*GSTF2) subunit was only detected at low levels in roots of untreated plants, but was strongly expressed in roots and above-ground organs following treatment with the safener dichlormid (Holt et al. 1995).

GSTs induced in response to safeners, herbicides, and pathogens have been identified and biochemically characterized in wheat (Mauch and Dudler 1993; Cummins et al. 1997; Riechers et al. 1997; Pascal et al. 1998; Pascal and Scalla 1999; Theodoulou et al. 2003). Our studies have utilized the diploid wheat *Triticum tauschii* (synonymous with *Aegilops tauschii* and *Aegilops squarrosa*), which is considered to be the D-genome donor to cultivated, hexaploid bread wheat *Triticum aestivum*. *Triticum tauschii* thus serves as a useful model plant and genome to understand regulation of GST expression in grass crops with large and/or polyploid genomes (Keller and Feuillet 2000; Xu et al. 2002). Our previous research focused on a herbicide safener-induced GST isozyme that was purified from *T. tauschii* (Riechers et al. 1997). This safener-inducible GST isozyme can use the chloroacetamide herbicide dimethenamid as a substrate (Riechers et al. 1997), where its conjugation with reduced glutathione results in metabolic detoxification of the herbicide (Dixon et al. 1998). This same GST protein, which is also found in *T. aestivum*, was recently characterized and its crystal structure determined (Thom et al. 2002). Subsequent molecular studies in our laboratory showed that there are actually two safener-induced GSTs that are encoded by a tandem duplication of the gene in *T. tauschii* (Xu et al. 2002). These genes are highly induced by safener treatment in etiolated shoots. The objective of our current study was to examine GST expression profiles in dissected coleoptiles and new leaves of etiolated *T. tauschii* shoots, with and without safener treatment. Our results provide new evidence for tissue-specific expression of GST transcripts and proteins in etiolated shoots; we also report for the first time on the subcellular localization of constitutive and safener-induced GST proteins in *T. tauschii* shoots.

Materials and methods

Plant material

For RNA and protein extraction and analysis, seeds were planted in plastic pots containing vermiculite. Pots were watered to saturation with deionized water, 10 μM cloquintocet-mexyl, or 10 μM fluxofenim, covered with aluminum foil, and subjected to prechilling at 4 °C for 5 days to increase and synchronize seed germination. Pots were then removed from the cold and incubated at room temperature without light for 5 days. Etiolated shoots were harvested, coleoptiles were dissected away from the new leaves, and both tissues were frozen in liquid nitrogen and stored at −80 °C until RNA or protein extraction.

Total RNA and protein extraction

Total RNA extraction, RNA gel-blot analysis, and semiquantitative RT–PCR techniques were performed exactly as described previously (Xu et al. 2002). Protein extractions were performed at

10 °C. Total soluble proteins were extracted from 0.5 g etiolated shoots, or dissected coleoptiles or new leaves, in 1.5 ml of buffer containing 200 mM Tris–HCl (pH 7.8), 5 mM 2-mercaptoethanol, 1 mM Na_2EDTA, and 50 mg/ml polyvinylpolypyrrolidone, using a glass tissue homogenizer. Crude protein extracts were clarified by centrifugation at 12,000 *g* for 5 min, and the supernatant was removed and used for GST activity assays or immunoblot analyses. Proteins were quantified using a commercially available kit (Bio-Rad, Hercules, CA, USA) based on the Bradford method (Bradford 1976), using bovine serum albumin as a standard.

Antibody production

A recombinant GST protein was generated using PCR primers to amplify the majority of the *TtGSTU1* coding region. The forward primer had the sequence 5′-GGA-GATGACCTGAAGCTGCTC-3′, the reverse primer had the sequence 5′-CTCTCGTGCCTTGGCAAAC-3′, and the *TtGSTU1* cDNA (formerly named *GST TSI-1*; GenBank accession AF004358) in pBluescript was used as template. The resulting PCR product (660 bp) was purified from a 1.2% agarose gel and ligated into the pBAD Thio/TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). The resulting plasmid was purified and sequenced on both strands to ensure that the GST coding region was in the correct reading frame for subsequent expression. The recombinant protein was expressed in *Escherichia coli* following induction of the culture with arabinose, affinity-purified, and digested with enterokinase according to the manufacturer's instructions. The approximate 27-kDa GST protein was gel-purified by SDS–PAGE and was subsequently used for antibody generation. New Zealand White rabbits were immunized by subcutaneous injection using 0.5 mg GST protein for primary and secondary injection and 0.25 mg for all subsequent injections. Immunizations consisted of 1 ml volumes of a 1:1 emulsification of the antigen into Titermax Gold adjuvant (Sigma-Aldrich, St. Louis, MO, USA) for the primary injection and Freund's incomplete adjuvant for all subsequent booster injections. Antigen was administered every 3 weeks, and polyclonal antiserum was collected the fourth and eighth weeks after injection for immunoassay screening. Whole blood was collected in a centrifuge tube after nicking the marginal ear vein of the rabbit. The blood was allowed to clot at room temperature and was centrifuged to sediment the cellular fraction. The serum was drawn off and frozen at −20 °C until used for immunoblot analysis.

In addition to the recombinant GST protein, a synthetic peptide was also utilized for antibody generation. The peptide sequence QWLRGKTEEEKSEGKKQA (corresponding to position 115–132 in the *Tt*GSTU1 protein; Xu et al. 2002) was chosen due to its high potential for immunogenicity (i.e., many charged amino acids), as determined by the MacVector software program. This 18-amino-acid peptide is encoded by the beginning of Exon 2 of the *TtGSTU1* genomic sequence, which was noted to be part of a strong hydrophilic region of the protein (Xu et al. 2002). A cysteine residue was added to the C-terminus of the peptide sequence to aid in conjugation to the carrier protein, and was synthesized by the Protein Sciences Facility, University of Illinois, Urbana. Conjugation of the peptide was performed using Sulfo-SMCC cross-linker and keyhole limpet hemocyanin as the carrier protein. The resulting conjugate was dialyzed into phosphate-buffered saline and used as the immunogen for injecting rabbits as described above.

Electrophoresis and immunoblot analysis

One-dimensional electrophoresis (SDS–PAGE) was conducted according to the method of Laemmli (1970) using 17.5% acrylamide/bis (37.5:1) minigels (Bio-Rad). Gels were run at 150 V for 2 h. For immunoblot experiments, gels were equilibrated in transfer buffer [48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% (w/v) SDS] for 30 min, then electrophoretically transferred to 0.45 μm nitrocellulose membranes using a semidry transfer apparatus (Trans-Blot SD; Bio-Rad) operating at 15 V for 50 min. Membranes were then incubated in blocking buffer I [20 mM Tris–HCl (pH 7.5), 180 mM NaCl, 5% (w/v) nonfat dry milk] for 3 h before being probed overnight with the primary antiserum. Primary antisera were diluted 1:20,000 for the recombinant GST antiserum, or diluted 1:10,000 for the synthetic peptide antiserum, in blocking buffer II [20 mM Tris–HCl (pH 7.5), 180 mM NaCl, 3% (w/v) nonfat dry milk, 0.1% (v/v) Tween 20]. Blots were washed with wash buffer [20 mM Tris–HCl (pH 7.5), 180 mM NaCl, 0.1% Tween 20] 4 times for 15 min per wash. Blots were then incubated for at least 2 h with secondary antibody (anti-rabbit IgG conjugate to alkaline phosphatase; Sigma) at a dilution of 1:6,000 in blocking buffer II. Blots were washed again 4 times with wash buffer, then developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as alkaline phosphate substrates.

GST activity assays

GST activity assays, using the herbicide dimethenamid as a substrate, were conducted essentially as described previously (Riechers et al. 1996b). Crude protein extracts (100 μl) from dissected coleoptiles or new leaves were used for the assays. GST enzyme activity is expressed as pmol of dimethenamid–glutathione conjugate formed per minute, after subtracting the nonenzymatic conjugation rate from each enzyme-catalyzed reaction. The experiment was conducted twice, with three replications per treatment.

Microscopy and immunocytochemistry

Standard electron microscopic procedures Preparation of samples for standard electron microscopy is modified from a basic protocol described by Vaughn et al. (1990)

that works well with a variety of difficult-to-fix plant materials. Samples of leaf and coleoptile tissue were cut into small segments with a razor blade in a pool of 6% glutaraldehyde in 0.05 M Pipes buffer (pH 7.4) on dental wax, and the samples transferred to a vial of the same liquid for 2 h at room temperature. Rings of tissue were cut so that the coleoptile and encompassed leaf tissue would be held together during the fixation and processing. The samples were washed twice with 0.1 M cacodylate buffer (pH 7.2), 15 min each, and then post-fixed in 2% osmium in 0.1 M cacodylate buffer for 2 h. The specimens were washed several times in distilled water and en bloc stained with 2% uranyl acetate overnight at 4 °C. The specimens were washed extensively in distilled water and then dehydrated in an acetone series. Samples were transferred to propylene oxide prior to the addition of resin. Embedding, sectioning, post-staining and microscopic examinations were performed as described previously (Vaughn et al. 1990).

Immunocytochemical localizations Procedures were modified from a published protocol for the handling of dodder (*Cuscuta pentagona*) haustoria specimens for immunocytochemistry (Vaughn 2003). For immunocytochemical observations, specimens were cut in a pool of 3% glutaraldehyde in 0.05 M Pipes buffer (pH 7.4) on dental wax and transferred to vials of the same solution for 1–2 h at room temperature. As with the standard microscopic procedures, rings of tissue containing the coleoptile and encompassed leaf tissue were cut so that they would remain together during the fixation and embedding protocol. The samples were washed with Pipes buffer at 4 °C for two exchanges of 15 min each. Dehydration was carried out in cold ethanol, by increases in the concentration of the ethanol to 75% (v/v) in the Pipes buffer at 4 °C. After two 15-min exchanges of 100% ethanol at 4 °C, the samples were transferred to a −20 °C freezer and addition of L.R. White resin was made at 25% increments over a period of 4 days. After 24 h in 100% resin at −20 °C, the samples were allowed to warm to room temperature and the samples were shaken on a gyratory shaker for 24 h to enhance resin penetration. Samples were transferred to BEEM capsules (Polysciences, Warrington, PA, USA) and the capsules filled with fresh resin. Polymerization was accomplished at 50 °C in a vacuum oven in a time period of 2–4 h, depending upon the resin batch.

Semi-thin (0.35 μm) sections were cut with Delaware Diamond Knife histological diamond and were mounted to clean, chrome-alum-coated microscope slides on a slide-warming tray. Sets of serial sections were encircled with a black wax pencil to form a staining area for the subsequent incubation steps. Some sets of sections were stained with 1% Toluidine Blue in 1% sodium borate for 2 min, to access tissue preservation and orientation. The sections for immunological analysis were then treated as follows: 1% bovine serum albumin (BSA) in 0.02 M phosphate-buffered saline (pH 7.2) (PBS–BSA), 30 min; GST antiserum diluted from 1:80 to 1:800 in PBS–BSA, 3 h; PBS–BSA, four exchanges, 2.5 min each; 15-nm Protein A–gold (EY Labs, San Mateo, CA, USA) diluted 1:20 in PBS–BSA, 30 min; two exchanges of PBS, 2.5 min each. These reactions were performed in a smoked acrylic chamber with humidity near 100%. The samples were then washed extensively in distilled water to remove residual chloride and phosphate ions that might precipitate the silver reagents. To intensify the gold particles, the Amersham IntenSE reagents were mixed 1:1 and the samples allowed to react for 15–30 min in the chamber. The slides were washed extensively with distilled water and then mounted in Permount to make the slides permanent. Some samples were stained with Toluidine Blue to allow anatomical details of the specimen so as to determine the precise distribution of the immuno-reactive areas. Controls included samples that were pre-treated with 6% (w/v) sodium *m*-periodate to oxidize vicinal-OH groups in polysaccharides and then probed with GST antisera, samples that were probed with a polyclonal arabinogalactan protein antiserum (diluted 1:80) instead of the GST specific sera, or samples that were probed with a mixture of non-immunized rabbit serum from three animals (diluted 1:80) to serve as a preimmune serum. Specimens were observed and photographed with a Zeiss Axiophot microscope without the use of orange filters so that the Toluidine Blue areas would appear less intense than the silver-stained areas. Experiments have been repeated five times on three different sets of treated and untreated material. Serial sections from the same block face were used to compare the labeling pattern of the two GST antisera.

Results

Constitutive and safener-inducible GSTs are predominantly expressed in the coleoptile

Previous studies in our laboratory have shown that GSTs are highly induced in etiolated shoots of *Triticum tauschii* following safener treatment (Riechers et al. 1998; Xu et al. 2002). To further examine GST expression, coleoptiles and new leaves were dissecting out from etiolated shoots to see if differences could be found in tissue expression profiles. Total RNA was extracted from coleoptiles and new leaves, and RNA gel-blot analysis revealed that all of the constitutive GST expression is occurring in the coleoptile, while both coleoptiles and new leaves show induction of GST transcripts in response to the safeners cloquintocet-mexyl and fluxofenim (Fig. 1a). However, the highest amount of GST expression was observed in safener-treated coleoptiles, with fluxofenim causing the largest induction of GST transcripts (Fig. 1a).

Because we had previously shown that there are actually two tandemly duplicated GST genes in

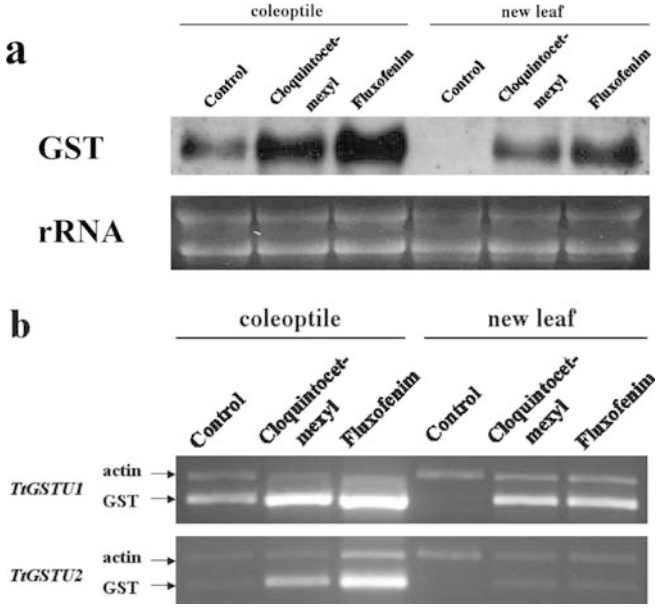

Fig. 1a, b Analysis of GST expression in dissected, etiolated *Triticum tauschii* shoots. Safener treatments were 10 μM cloquintocet-mexyl or 10 μM fluxofenim applied as vermiculite drenches. **a** *Upper panel* RNA gel-blot analysis of steady state GST mRNA levels in dissected coleoptiles and new leaves of *T. tauschii* shoots. The blot was probed with a *TtGSTU1* cDNA coding region probe. *Lower panel* Equal loading of total RNA per lane is indicated by ethidium bromide staining of ribosomal RNA bands in the gel before blotting. **b** Semi-quantitative RT–PCR analysis of individual *TtGSTU1* and *TtGSTU2* transcripts using gene-specific primers; methods were exactly as described in Xu et al. (2002). The wheat actin transcript serves as a constitutive, internal control to normalize expression levels among PCR samples and treatments

T. tauschii (*TtGSTU1* and *TtGSTU2*; Xu et al. 2002), semiquantitative RT–PCR was utilized to examine individual GST transcripts in control and safener-treated shoot tissues. As shown in Fig. 1a, when examining GST transcripts by RNA gel-blot analysis, no constitutive expression of either gene was detected in new leaves. The majority of constitutive expression in coleoptiles was contributed by *TtGSTU1* (Fig. 1b); this gene was also the most highly expressed in safener-treated coleoptiles. Both genes showed increased expression levels in response to both safeners in coleoptiles and new leaves, but *TtGSTU1* expression was always higher than that of *TtGSTU2* (Fig. 1b), as had been reported previously when examining whole shoots and roots (Xu et al. 2002).

In order to examine gene expression at the protein level, two different antisera were generated against the same tau class *Tt*GSTU1 protein: an antiserum generated against the entire recombinant GST protein, and an antiserum generated against a synthetic peptide derived from an internal region of *Tt*GSTU1. Plant GSTs are encoded by large multigene families and are comprised of many isozymes and subunits (McGonigle et al. 2000; Dixon et al. 2002; Wagner et al. 2002) and many tau class GSTs are found in wheat (Cummins et al. 1997; Dixon et al. 1998; Edwards and Dixon 2000), so a synthetic peptide antiserum was generated in an attempt to gain more specificity towards the safener-induced proteins *Tt*GSTU1 and *Tt*GSTU2. Immunoblot analysis was conducted to determine the specificity of these two antisera, using wheat crude protein extracts (Fig. 2). The *T. aestivum* 'Chinese Spring' 6DL ditelosomic aneuploid line was utilized because this line is missing the short arm of chromosome 6D, which contains the tandemly duplicated *TtGSTU1* and *TtGSTU2* genes (Riechers et al. 1998; Xu et al. 2002). When probing with the recombinant GST antiserum, increases in immunoreactive GST protein were observed in safener-treated *T. tauschii*, *T. aestivum*, and the *T. aestivum* 6DL aneuploid (Fig. 2a). The observation that constitutive and safener-induced GSTs were recognized by this antiserum in the 6DL aneuploid line demonstrates that this antiserum is detecting GST proteins other than *Tt*GSTU1 and *Tt*GSTU2. In contrast, the increased specificity of the synthetic-peptide antiserum is demonstrated by the low constitutive immunoreactive GST protein (lanes 1, 3 and 5), the large increase in immunoreactive GST protein in safener-treated *T. tauschii* and *T. aestivum*, and the minimal immunoreactivity noted in the chromosome 6DL aneuploid line (Fig. 2b).

These two antisera were also utilized to investigate GST protein expression in dissected coleoptiles and new leaves of *T. tauschii* (Fig. 3). When probing with the recombinant GST antiserum, the relatively high amount of constitutively expressed immunoreactive GST protein in both coleoptiles and new leaves is inconsistent with the results of RNA gel-blot analysis and RT–PCR (Fig. 1). This indicates that the recombinant GST antiserum is recognizing other tau-class GST proteins, in addition to *Tt*GSTU1 and *Tt*GSTU2, yet this antiserum also detects increases in GST levels in both tissues (Fig. 3a). The greater specificity of the synthetic peptide antiserum is once again noted by comparing the results of the immunoblot (Fig. 3b) with the corresponding results of the RNA gel-blot (Fig. 1a). No constitutive

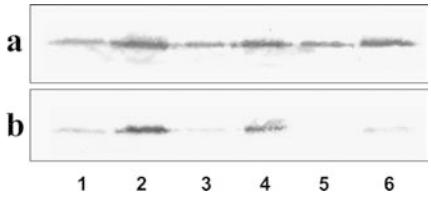

Fig. 2a, b Immunoblot analysis to determine the specificity of the two GST antisera generated against the same *Tt*GSTU1 protein. Separate immunoblots were probed with the recombinant GST antiserum (**a**) or the synthetic-peptide antiserum (**b**). Safener treatment was 10 μM cloquintocet-mexyl applied as a vermiculite drench. All lanes in each blot contained 9 μg of total soluble protein extracted from etiolated wheat shoots. *Lane 1*, *T. tauschii* control; *lane 2*, *T. tauschii* treated with cloquintocet-mexyl; *lane 3*, *T. aestivum* 'Chinese Spring' control; *lane 4*, *T. aestivum* 'Chinese Spring' treated with cloquintocet-mexyl; *lane 5*, *T. aestivum* 'Chinese Spring' chromosome 6DL aneuploid line, control; *lane 6*, *T. aestivum* 'Chinese Spring' chromosome 6DL aneuploid line, treated with cloquintocet-mexyl. The protein band recognized by the antiserum in each blot is approximately 27 kDa

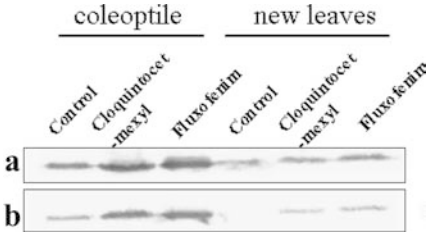

Fig. 3a, b Immunoblot analysis of GST expression levels in dissected coleoptiles and new leaves of etiolated *T. tauschii* shoots. Separate immunoblots were probed with the recombinant GST antiserum (**a**) or the synthetic-peptide antiserum (**b**). Safener treatments were 10 μM cloquintocet-mexyl or 10 μM fluxofenim applied as vermiculite drenches. All lanes in each blot contained 9 μg of total soluble protein extracted from etiolated shoots. The protein band recognized by the antiserum in each blot is approximately 27 kDa

expression was detected in new leaves, yet both coleoptiles and new leaves showed safener induction of immunoreactive GST protein. In addition, a higher level of GST protein was noted in safener-treated coleoptiles relative to new leaves (Fig. 3b).

GST herbicide-metabolizing activity is predominantly located in the coleoptile of safener-treated shoots

Very little GST activity was detected in either coleoptiles or new leaves of untreated shoots (Table 1). However, both cloquintocet-mexyl and fluxofenim caused dramatic increases in GST enzymatic activity in dissected coleoptiles, relative to the untreated control. Fluxofenim induced a greater increase in total GST activity in the coleoptile, relative to cloquintocet-mexyl, which has been reported previously with intact wheat shoots (Riechers et al. 1996b). Both safeners caused a similar increase in total GST activity in new leaves (Table 1), although this activity was several-fold less than that measured in coleoptiles.

Both total GST activity and specific GST activity were greater in coleoptiles than in new leaves; however, there was a much higher specific GST activity in coleoptiles in response to safener treatment due to the lower protein concentrations (Table 1). Coleoptiles contain about 5-fold less total soluble protein on a per gram fresh weight basis than the new leaves, yet also have more total GST activity, which results in a much greater GST specific activity in the coleoptile.

GST proteins are localized predominantly to the epidermal and sub-epidermal cell layers of safener-treated coleoptiles

In young *T. tauschii* seedlings, the coleoptile encloses the developing leaves in a protective layer of tissue. The cytological differences between the coleoptile and leaf tissues are striking, with a majority of the coleoptile cells containing only a large vacuole and a thin rim of underdeveloped organelles in the cytoplasm appressed to the cell wall (Fig. 4a). In contrast, the cells of the young leaf are filled with organelles and contain many etioplasts, with prominent prolamellar bodies (not shown). When seedlings are treated with the safener fluxofenim (10 μM), few changes are noted in the structure of these tissues with the exception of the vacuoles, especially in both the epidermal and sub-epidermal tissues of the coleoptile. Electron-opaque aggregates are now prominent within these vacuoles (Fig. 4a) and the tonoplast is deeply invaginated with bits of cytoplasm that appear to be recently engulfed into the vacuole (Fig. 4b). Serial sections of these invaginations reveal that some are connected to the cytoplasm through long stretches of appressed tonoplast (arrowheads in Fig. 4a) whereas others are truly engulfed in the vacuole. In the untreated seedlings, only very fine fibrillar aggregates are observed in the vacuoles of either leaves or coleoptiles (not shown). The vacuolar invaginations noted in fluxofenim-treated tissues are rare or entirely absent in control tissues.

When semi-thin sections of fluxofenim-treated coleoptiles were probed with the antiserum raised to the recombinant GST, a strong reaction was noted in the epidermal and sub-epidermal layers of the coleoptile, with a weaker reaction in other coleoptile and leaf cells (Fig. 5a, d). When the sections are post-stained with Toluidine Blue, details of the tissues and cells are obvious as paler staining areas and the immuno-reactions as more strongly stained areas, appearing somewhat in relief. A thin rim of reaction occurs along the cytoplasm in the epidermal cells (Fig. 5a). Larger aggregates are

Table 1 GST enzyme activity in dissected coleoptiles and new leaves of etiolated *Triticum tauschii* shoots, measured with the herbicide dimethenamid as the substrate. Mean values for GST activity and protein concentration are reported (±SE)

Tissue	Safener treatment	Total activity[a] (pmol min^{-1} g FW^{-1})	Specific activity (pmol min^{-1} mg $protein^{-1}$)	Total protein[c] (mg ml^{-1})
Coleoptile	Untreated	8.0 (6.4)	8.5 (7.5)	0.61 (0.06)
	10 μM Cloquintocet	171.2 (16.0)	154.3 (15.0)	0.69 (0.01)
	10 μM Fluxofenim	420.8 (14.4)	396.2 (31.2)	0.68 (0.08)
New leaves	Untreated	ND[b]	ND	2.95 (0.23)
	10 μM Cloquintocet	86.4 (11.2)	16.6 (2.3)	3.24 (0.07)
	10 μM Fluxofenim	75.2 (8.0)	17.1 (2.7)	2.89 (0.53)

[a]GST enzyme activity values are corrected for non-enzymatic conjugation rates
[b]Not detectable
[c]Protein concentration in the crude extract

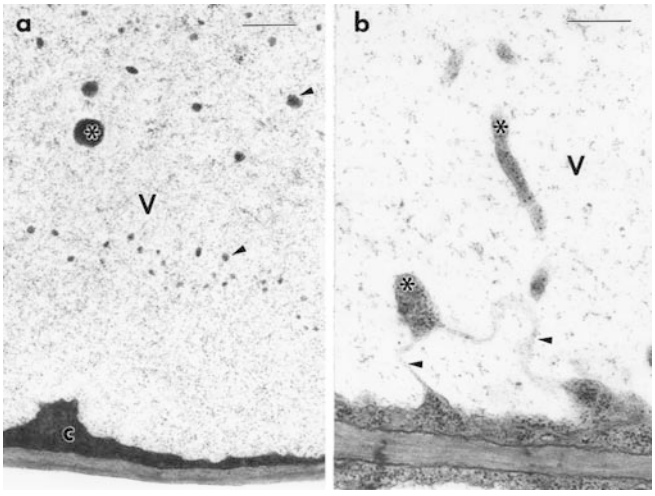

Fig. 4a, b Transmission electron micrographs revealing the ultrastructure of the vacuoles of fluxofenim-treated *T. tauschii* coleoptiles. **a** Sub-epidermal cell with a thin rim of cytoplasm (*C*) surrounding a large vacuole (*V*). Within the vacuole, an amorphous electron-opaque accumulation (*arrowheads*), accompanied by larger masses of electron-dense deposits, is noted with an *asterisk* (*). **b** Although the cytoplasm of the coleoptile cells is relatively appressed to the cell wall and bound by the vacuole (*V*), small invaginations of the cytoplasm are found in the vacuole (*). Some of these are no longer in contact with the bulk of the cytoplasm as revealed by serial sections, whereas others reveal limited contact with appressed regions of the tonoplast membrane (*arrowheads*). Bars = 0.5 μm

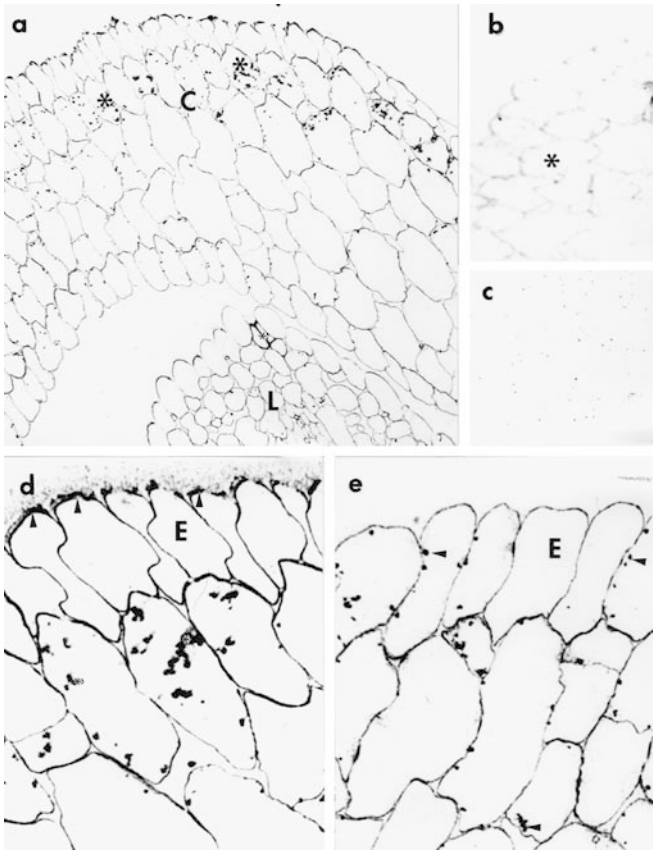

Fig. 5a–e Light micrographs of immunogold–silver localizations of GSTs in coleoptiles and developing leaves of fluxofenim-treated (**a, b, d, e**) and unsafened (**c**) *T. tauschii* shoots, using the recombinant GST antiserum (**a, d**) and the antiserum raised to the synthetic peptide (**c, e**). **a** Low-magnification micrograph of the coleoptile (*C*) and the leaf (*L*), which is encompassed by the coleoptile, and probed with the recombinant GST antiserum. The outer epidermis and the adjacent sub-epidermal cells are the most strongly reactive tissue areas, with a strong reaction in the epidermal cytoplasm and the vacuoles of the sub-epidermal cells (* in C). Reaction in the leaf is relatively low, with the exception of a putative developing guard cell (* in L). Paler areas on the section are from the reaction with Toluidine Blue to reveal general structure. **b** Micrograph of the coleoptile probed with a combined pre-immune serum (diluted 1:80) from three rabbits, and lightly post-stained with Toluidine Blue. No reaction is noted, even in the sub-epidermal layer (*) that reacted strongly with the recombinant GST antiserum. **c** Section of coleoptile tissue from seedlings without safener treatment (and without Toluidine Blue post-staining), probed with the synthetic peptide antiserum. Only a background reaction is noted in these unsafened tissues. **d** Higher magnification micrograph of cells in the epidermis and sub-epidermis of the coleoptile, probed with the recombinant GST antiserum. These sections reveal the massive reaction (*) present in the vacuoles of the sub-epidermal cells and the strong reaction along the edge of the epidermal (*E*) cells (*arrowheads*). **e** Serial section from the same block face as in **d**, but probed with the synthetic-peptide antiserum. Although the reactions are similar to those observed with the recombinant GST antiserum, some subtle differences exist. Reactions occur along the cytoplasm (*arrowheads*) in both epidermal (*E*) and sub-epidermal cells; however, the reaction in the epidermal cells is less intense than in **d**. In addition, reactions occur most strongly in the cytoplasm rather than throughout the vacuole in the sub-epidermal cells, as was observed in **d**. **a** ×160; **b**, **c** ×240; **d**, **e** ×320

found in the epidermal and sub-epidermal cell vacuoles (Fig. 5a, d), corresponding in position and shape to the vacuolar aggregates noted by transmission electron microscopy in this tissue (Fig. 4a). Cells on the outer edge of the coleoptile were much more reactive than those cells more proximal to the leaf tissue. Reaction in the leaf tissue was much weaker than the coleoptile, but an occasional putative developing guard cell reacted strongly (Fig. 5a). A weak reaction occurred in the leaf epidermal or mesophyll cells and these reactions were always cytoplasmic. Because polyclonal sera often contain epitopes that recognize polysaccharides or glycoproteins, some segments were treated with saturated sodium *m*-periodate prior to labeling to oxidize vicinal-OH groups on the polysaccharides. However, this pretreatment did not influence the labeling of the vacuolar aggregates with the GST antisera (not shown); similar negative results were obtained with an arabinogalactan protein antiserum, which also showed no vacuolar labeling, although as expected cell wall and plasma membrane reactions were noted with this serum (not shown). Likewise, probing of fluxofenim-treated coleoptile sections with a mixture of serum from three non-immunized rabbits resulted in no reaction (Fig. 5b). These data indicate that the immuno-reactive substance in the vacuole is from neither polysaccharide nor arabinogalactan protein sources, nor something that reacts generally with non-immunized rabbit serum. Thus, whatever the nature of the vacuolar reaction, it is one related to the action of the safener.

In contrast to the labeling pattern of the recombinant GST antiserum, probing with the synthetic peptide antiserum labeled only the epidermal and sub-epidermal cells, and in these cells, only the thin rim of cytoplasm that surrounds the vacuole (Fig. 5e). The pattern of immuno-reaction in the epidermal cells is similar but less intense than that obtained with the recombinant GST antiserum (Fig. 5d). However, most of the reaction in the sub-epidermal cells appears to be confined to the peripheral cytoplasm, with only a few of the reactions labeling the vacuole.

These immunocytochemical data confirm the results from the dissected-tissue homogenizations that indicated the tissue-specific expression of the safener-induced GSTs is predominantly localized to the coleoptile (Figs. 1, 3; Table 1). When sections from control tissue (without safener treatment) were probed with the recombinant GST antiserum, no reactions in the vacuole were noted and the cytoplasmic reactions were much less prominent than in the safener-treated tissues (not shown). Cellular reactions were not observed in tissues without safener treatment when probing with the synthetic peptide antiserum (Fig. 5c). These differences in immunoreactivity do not appear to be due to the titer of the primary antisera, as increases in the synthetic peptide antiserum to a 1:20 dilution revealed no tissue staining beyond background (not shown). However, at these high antiserum concentrations, the background reaction over acellular areas was also very high.

Discussion

GST expression was examined by measuring mRNAs, protein abundance, enzymatic activity with a herbicide substrate, and immunoreactive protein localization in dissected coleoptiles and new leaves of etiolated *Triticum tauschii* shoots. Our data support the hypothesis that the coleoptile is a critical organ in protecting the newly developing leaves of grass shoots from chloroacetamide herbicide injury (Fuerst et al. 1991). Chloroacetamide herbicides, such as dimethenamid, are typically applied to the soil before or at the same time that grass crops are planted, and have been shown to inhibit elongase enzymes that are involved in the biosynthesis of very long chain fatty acids (Boger et al. 2000; Schmalfub et al. 2000). Herbicide safeners appear to exert their main effect on herbicide metabolism in the coleoptiles of etiolated shoots by inducing GSTs that rapidly detoxify the parent herbicide and prevent it from reaching the new leaves as the shoot emerges through the soil (Fuerst et al. 1991; Fuerst and Lamoureux 1992). The comparative data presented in this paper on GST expression in coleoptiles vs. new leaves provide new insight into the mechanism for the increased herbicide metabolism in the coleoptile in response to safener treatment. GST expression appears to be tightly controlled and regulated, as evidenced by the tissue- and cell-specific expression patterns observed in safener-treated *T. tauschii* shoots (Fig. 5), as well as the genome- and organ-specific expression patterns reported in hexaploid wheat (Xu et al. 2002). As reported previously, GST proteins in the foliage of major cereal crops have been estimated to occur at levels up to 2% of total soluble proteins (Dixon et al. 2002). Our data show that the vast majority of this GST protein is located in the epidermal and sub-epidermal cell layers of the coleoptile in safener-treated shoots.

Even within the coleoptile there is apparently a further specificity in the cell layers that respond to the safener treatments with enhanced levels of GST, as a majority of reaction product is detected in the outer epidermal cells and the adjacent sub-epidermal cells (Fig. 5). The two outer cell layers of the coleoptile would be in closest contact to the safener as the etiolated seedlings grow towards the surface. Thus, they might receive a relatively higher concentration of the safener compared to either the leaves or other tissue within the coleoptile, (i.e., the tissue distribution of the induced GST protein is related to a safener concentration gradient). However, another possibility is that these two cell layers are already biochemically equipped to deal with xenobiotics, or that they perform a metabolic function in the emerging seedling, such as flavonoid biosynthesis or protein turnover, that allows these tissues to respond in such a unique manner to safener treatment. For example, it has been well documented that a rapid tagging of phytochrome by ubiquitin, and subsequent movement to the vacuole, occurs in oat coleoptile tissues (Vierstra et al. 1985; Speth et al. 1987). The GST increases related to safener treatment and subsequent enhancement of herbicide metabolism may be but one of several biochemical functions these tissues perform in etiolated grass shoots.

The appearance of immunoreactive material in the vacuole of these tissues is another very intriguing but unresolved aspect of our cytological results. The recombinant GST antiserum clearly reacted with epitopes present throughout the vacuole, while the synthetic peptide antiserum labeling was mainly cytoplasmic (or possibly near the edge of the vacuole, but was difficult to discern). The variation in the vacuolar immunoreactions between the two antisera may be indicative of the more limited epitopes exposed to the synthetic peptide antiserum in the putative GST vacuolar aggregations. Alternatively, alteration of the specific epitopes in the region that are recognized by the synthetic peptide antiserum, due to vacuole incorporation of the GST protein (complexing with the safener and/or disruption of protein folding in the acid milieu of the vacuole), might have influenced the reactivity of the two antisera in our studies. Another possibility is that the recombinant GST antiserum recognizes a subset of tau-class GST isozymes that are involved with vacuolar accumulations in the coleoptile, but that these tau-class GSTs are not recognized by the synthetic peptide antiserum. Evidence for the presence of different tau-class GSTs in wheat was indicated by the antibody specificity results depicted in Fig. 2.

Because it is known that xenobiotic-glutathione conjugates follow a cellular pathway of flux from the cytosol into the vacuole, as shown using fluorescence microscopy techniques (Coleman et al. 1997), it is possible that the immunoreactivity of the GST antisera in the vacuole represents a novel form of "suicide" metabolism of the GST protein–safener–glutathione complex in the vacuole. This sort of a "suicide" detoxification mechanism has been reported previously with GST proteins in the liver of animals (Jakoby and Keen 1977), where GSTs were reported to sacrifice themselves by covalently binding to sufficiently toxic and electrophilic compounds. In plants, the BZ2 protein from maize is another tau-class GST protein that is involved in a similar form of vacuolar movement of anthocyanins (Walbot et al. 2000), although BZ2 protein was not directly localized inside the vacuole as with the tau-class GST proteins in our experiments. Our immunocytochemical observations and the proposed "suicide" detoxification mechanism for xenobiotics would also help to explain why such high levels of GST protein (1–2% of total soluble protein) are found in corn or wheat shoots treated with safeners (Dixon et al. 2002).

One intriguing, yet unresolved question about herbicide safeners is why they only protect grass crops and not dicot crops from herbicide injury (Davies and Caseley 1999). A recent study demonstrates that GSTs are induced by herbicide safeners in *Arabidopsis thaliana* (DeRidder et al. 2002), indicating that either tissue-specific GST expression in the coleoptile is critical for grasses, or that another component of the overall detoxification pathway is missing in dicots. Our data demonstrating the importance of the coleoptile for the safener response in grasses might help to explain this differential phenotypic response, since dicots do not have a comparable organ as their seedlings emerge from the soil. Other data in support of this theory is that safener-unresponsive *Triticum* species that lack the D genome, such as *T. monococcum* (AA) and *T. turgidum* subsp. *durum* (AABB), show much lower GST transcript levels in the shoot in response to safener treatment than *T. tauschii* (Xu et al. 2002).

Increased GST expression levels in response to safener treatment, when measuring mRNA, proteins, and enzymatic activity with a herbicide substrate, are consistent and indicate that the underlying mechanism for safener-enhanced GST expression is an increased rate of gene transcription. Future studies in our laboratory will examine the promoters of the safener-responsive *TtGSTU1* and *TtGSTU2* genes (Xu et al. 2002) to identify and functionally characterize the important *cis*-elements and *trans*-acting factors that are involved in enhanced levels of gene transcription. Additionally, immunocytochemical studies will help to reveal the function and roles of safener-inducible GST proteins in the overall herbicide detoxification pathway in grass crops and further explore the importance of the coleoptile in xenobiotic metabolism, using *T. tauschii* as a model system to study safener mechanism of action.

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