Proteomic Analysis of Arabidopsis Glutathione S-transferases from Benoxacor- and Copper-treated Seedlings

Aaron P. Smith¹,³,*, Ben P. DeRidder¹,⁴,*, Woei-Jiun Guo¹, Erin H. Seeley², Fred E. Regnier², and Peter B. Goldsbrough¹

¹Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907-1165
²Department of Chemistry, Purdue University, West Lafayette, IN 47907-2084
³Current Address: Department of Genetics, The University of Georgia, Athens, GA 30602
⁴Current Address: United States Department of Agriculture, Agricultural Research Service, Pacific West Area, 4135 E. Broadway, Phoenix, AZ 85040

*The first two authors contributed equally to this work

Corresponding author: Peter B. Goldsbrough

Tel: (765) 494-1334; Fax: (765) 494-0391; E-mail: goldsbrough@purdue.edu

Running title: Proteomic analysis of Arabidopsis glutathione S-transferases
Summary

Glutathione S-transferases (GSTs) are involved in many stress responses in plants, participating in the detoxification of xenobiotics and limiting oxidative damage. Studies examining the regulation of this gene family in diverse plant species have focused primarily on RNA expression. A proteomics method was developed to identify GSTs expressed in Arabidopsis seedlings and to determine how the abundance of these proteins changed in response to copper, a promoter of oxidative stress, and benoxacor, a herbicide safener. A total of eight GSTs were identified in seedlings grown under control conditions and only one of these, \textit{At}GSTU19, was induced by benoxacor. In contrast, four GSTs were significantly more abundant in copper-treated seedlings, \textit{At}GSTF2, \textit{At}GSTF6, \textit{At}GSTF7, \textit{At}GSTU19. The different responses to these treatments may reflect the potential for copper to impact many more aspects of plant growth and physiology compared to a herbicide safener. Differences between RNA and protein expression of GSTs indicate that both transcriptional and translational mechanisms are involved in regulation of GSTs under these conditions.
Introduction

Glutathione S-transferases (GSTs; or glutathione transferases) are a collection of multi-functional proteins that are found in essentially all organisms. In addition to their well known role as phase II detoxification enzymes (1), GSTs also provide protection against oxidative stress, catalyze various metabolic reactions, and serve as carrier proteins for a number of endogenous ligands (2, 3, 4, 5).

GSTs are grouped into distinct classes based on a number of criteria, including nucleotide sequence, gene structure, immunological cross-reactivity, kinetic properties, and structural characteristics (3). Some classes of GSTs are taxa-specific while others are found across kingdoms. Plant GSTs fall into one of six classes: phi, tau, theta, zeta, lambda, and glutathione-dependent dehydroascorbate reductases (DHARs). The two largest classes, phi and tau, are plant-specific, whereas the theta and zeta classes are also found in animals, fungi, and insects. To date, lambda and DHAR-type GSTs have been identified only in plants (5).

Many plant GSTs have been characterized as enzymes that catalyze the conjugation of glutathione (GSH) to a wide variety of xenobiotics, notably herbicides. Studies with compounds known as herbicide safeners, which are used to enhance herbicide tolerance of crop plants, have contributed to the understanding of this primary function of GSTs in plants (6). Safeners may increase herbicide tolerance by a number of mechanisms, but the bulk of the evidence indicates they selectively induce GSTs that detoxify specific herbicides. For example, the safener benoxacor protects maize from chloroacetanilide herbicides (e.g. metolachlor and alachlor) by increasing the expression of GSTs that efficiently conjugate these herbicides to GSH, but has no protective effect on dicotyledenous weeds (7).
As potent inducers of GST expression, safeners can help to elucidate the signaling pathways that govern the expression of plant GSTs involved in xenobiotic detoxification. A recent study demonstrated that RNA expression of several Arabidopsis GST genes is differentially induced by a number of safeners (8). These results suggest that induction of Arabidopsis GSTs by safeners is complex and occurs via multiple signaling pathways.

Aside from their role in xenobiotic detoxification, plant GSTs also provide protection against oxidative stress. Functioning as glutathione peroxidases (GPOXs), plant GSTs can catalyze the reduction of hydroperoxides to less harmful alcohols (9, 10). Expression of a GST with GPOX activity in transgenic tobacco provided protection against oxidative stress (11). Plant GSTs may also protect against oxidative stress by conjugating GSH to toxins produced as a result of oxidative damage to endogenous compounds. For example, heavy metals (e.g. copper) can induce oxidative stress, resulting in membrane lipid peroxidation and the formation of cytotoxic alkenals (12). One such alkenal, 4-hydroxynonenal, is a substrate for phi-class GSTs from sorghum (13), and wheat tau-class GSTs exhibit similar activities (14).

In addition to their roles in xenobiotic detoxification and oxidative stress tolerance, plant GSTs also catalyze metabolic isomerization reactions. For example, AtGSTZ1 participates in tyrosine catabolism in Arabidopsis by catalyzing the cis-trans conversion of maleylacetooacetate to fumarylacetooacetate (4). Finally, some plant GSTs function as nonenzymatic binding proteins of endogenous compounds such as anthocyanins and phytohormones (2, 15, 16).

The GST gene family in Arabidopsis includes 53 genes (5, 17). Given the diverse functions of GSTs in plants, it is not surprising that a wide range of factors induce RNA expression of these genes, including herbicides, heavy metals, extreme temperatures, phytohormones, and pathogen attack (2, 3). Although an abundance of data has been gathered.
on RNA expression of many of these GSTs, much less is known about the regulation of GST
protein expression. Therefore, the objectives of this study were to identify the GSTs expressed
in Arabidopsis seedlings and to quantify the impact of various environmental conditions on GST
protein expression. Because two major functions of plant GSTs are detoxification of xenobiotics
and protection against oxidative stress, we examined the expression of Arabidopsis GSTs in
response to a herbicide safener, benoxacor, and an inducer of oxidative stress, copper—conditions that address these two important functions of plant GSTs. Because of the
paucity of information about GST protein expression, a proteomic approach was used to examine
multiple GST proteins simultaneously.

Using a method adapted from the signature peptide approach (18, 19), eight GSTs were
identified in Arabidopsis seedlings: \( \text{AtGSTF2, AtGSTF6, AtGSTF7, AtGSTF8, AtGSTF9,} \)
\( \text{AtGSTF10, AtGSTFU19, and AtGSTU20.} \) The herbicide safener benoxacor induced
accumulation of \( \text{AtGSTU19,} \) whereas copper treatment resulted in higher levels of \( \text{AtGSTF2,} \)
\( \text{AtGSTF6, AtGSTF7,} \) and to a lesser extent, \( \text{AtGSTU19.} \) In contrast, \( \text{AtGSTF9} \) was significantly
less abundant in response to both treatments. Although RNA expression generally reflected the
changes in abundance of GST proteins in response to benoxacor and copper, transcript levels of
\( \text{AtGSTF8, AtGSTF9, and AtGSTF10} \) were notable exceptions.

**Experimental procedures**

**Protein extraction and purification**

Seeds of *Arabidopsis thaliana* ecotype Columbia were germinated in half-strength MS
liquid medium, grown for 9 days, treated for 24 hr with 100 µM benoxacor (Syngenta,
Greensboro, NC, USA) or 50 µM \( \text{CuSO}_4 \), harvested, weighed, frozen in liquid nitrogen, and
stored at -70° C. Seedlings (75-100 g) were ground to a fine powder in liquid nitrogen using a chilled mortar and pestle and homogenized in 3 volumes (w:v) of extraction buffer (50 mM Tris-HCl, pH 8; 5 mM DTT; 1 mM EDTA) using a Cyclone IQ² microprocessor system (Virtis, Gardiner, NY, USA). After filtration through 8 layers of cheesecloth and centrifugation at 12,000 g for 15 min, the supernatant was brought to 80% saturation with solid ammonium sulfate ((NH₄)₂SO₄), and centrifuged at 12,000 g for 20 min. The pellets were resuspended in a small volume of saturated (NH₄)₂SO₄, and stored at -20° C. The precipitated protein was centrifuged at 12,000 g for 10 min and then dissolved in approximately 2 volumes of PBS buffer containing 5 mM DTT. Protein samples were dialyzed (Spectra/Por 12,000-14,000 MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) for 2 hours against 1 L PBS containing DTT with one change of buffer. The samples were centrifuged at 12,000 g for 20 min to remove undissolved material. The proteins were applied to a GSH-agarose affinity column (G-4510, Sigma, St. Louis, MO, USA; 5 mL bed volume) at a flow rate of 0.5 mL min⁻¹, followed by a wash (5 column volumes) with PBS buffer at a flow rate of 1 mL min⁻¹. Bound proteins were eluted with 20 mL of elution buffer (50 mM Tris-HCl, pH 7.5; 10 mM GSH) at a flow rate of 1 mL min⁻¹. Eluted proteins were concentrated and dialyzed against 0.1 M HEPES buffer, pH 8.0 using Amicon Ultra centrifugal filter devices (Millipore Corp., Bedford, MA, USA; 10-kDa cutoff). Protein samples were kept at 4° C or on ice at all times and protein concentrations were determined using BSA as a standard (20). Immunoblot analyses were carried out as described using polyclonal antibodies against *At*GSTF2 or *At*GSTU19 (21).

**Trypsin digestion and peptide acetylation**

Protein samples obtained by GSH-affinity chromatography were diluted with 100 mM HEPES buffer, pH 8.0, containing 9 M urea to give a final urea concentration of between 7M and
Cysteine sulfhydryls were reduced by the addition of 10 mM DTT followed by incubation at 37° C for 2 hr. Free sulfhydryl groups were alkylated with iodoacetic acid (final concentration 20 mM) in the dark on ice for 2 hr. The alkylation reaction was quenched by addition of 10 mM cysteine followed by incubation on ice for 5 min. Samples were diluted with 0.1 M HEPES, pH 8.0, to a final urea concentration of 1 M. One µg sequencing-grade modified trypsin (Promega, Madison, WI, USA) was added for every 50 µg of protein in the sample. Proteolysis was carried out at 37° C for 12 hr and then quenched with 1 mg Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK). One mg acetoxysuccinamide was added to the control samples and 1 mg of d3-acetoxysuccinamide, synthesized as previously described (18), was added to the experimental samples. The samples were stirred at room temperature for 4 hr and each experimental sample (i.e. derived from either benoxacor- or copper-treated tissue) was combined with its respective control sample. To reverse the acetylation of hydroxyl groups on tyrosine residues, 2 mg hydroxylamine hydrochloride was added to each mixture. The pH was adjusted to 12 with 5 M NaOH and the mixtures were stirred at room temperature for 10 min. Glacial acetic acid was added to adjust the pH to 8.0, and the samples were dried in a centrifuge dryer.

**Reverse-phase liquid chromatography and mass spectrometry**

The acetylated tryptic peptides were dissolved in 500 µL water and separated over a Microsorb-MV 100-5 C18 reverse-phase column (Varian, Palo Alto, CA, USA), with water and acetonitrile (ACN) (both containing 0.1% trifluoroacetic acid). Peptides were eluted at a flow rate of 1 mL min⁻¹ using a gradient of 15 % ACN to 60 % ACN over 25 min, followed by a step elution to 100 % ACN over 5 min. Thirty fractions of 1 mL were collected, dried in a centrifuge dryer, and dissolved in 100 µL of 50 % (v:v) methanol:water containing 0.1 % TFA. These samples were injected into a Q-Star electrospray ionization quadrupole time-of-flight (ESI-Q-
TOF) mass spectrometer (Perseptive Biosystems Inc., Framingham, MA, USA) at 8 µL min⁻¹. Masses were scanned from m/z 300 to 2,000 at default voltage potentials and abundant peptides were fragmented by restricting the ion gate voltage. Peptide parent ion masses were analyzed with Mascot (http://www.matrixscience.com). Mascot parameters included proteolysis by trypsin/chymotrypsin with one missed cleavage, alkylation of cysteine residues with iodoacetic acid, and size tolerances of 0.5 Da for peptides and 0.2 Da for peptide fragments. Variable modifications included sodiated (C-terminal), sodiated (D/E), acetyl heavy (K), acetyl heavy (N-terminal), acetyl light (K), and acetyl light (N-terminal). The relative abundance of GST peptides in control and benoxacor- or copper-treated samples was calculated as the ratio of their respective monoisotopic peak intensities.

**Isolation and analysis of RNA**

Total RNA was isolated from Arabidopsis seedlings, separated by electrophoresis through formaldehyde-agarose gels, transferred to a nylon membrane, UV cross-linked, and hybridized with ³²P-labeled cDNAs as previously described (21).

For real-time PCR analysis of the transcript for the copper-induced protein encoded by At2g28540, RNA was first treated with DNase (DNA-free™, Ambion, Austin TX). Complementary DNA was synthesized in a 30 µL reaction containing 2 µg RNA, 500 ng oligo-dT, 0.5 mM dNTPs and 1 µL AMV reverse transcriptase (Promega, Madison, WI) After incubation at 42°C for 50 min., the reverse transcriptase was inactivated by incubation at 70°C for 10 min. The cDNA was diluted to 600 µL and 5 µL was used as template for PCR. The gene specific primers for At2g28540 were 5'-ACCACCTGGCTTTTCAGTT and 5'-TCTGCATAGACAAAGGGTT. Expression levels were normalized using a tubulin gene (At5g12250) using the following primers: 5'-TGGGAACTCTGCTCATATCT and 5'-TCTGCATAGACAAAGGGTT. Expression levels were normalized using a tubulin gene (At5g12250) using the following primers: 5'-TGGGAACTCTGCTCATATCT and 5'-
GAAAGGAATGAGGTTCACTG. Real-time PCR was performed using an iCycler iQ (Bio-Rad, Hercules, CA) with SYBR I as the fluorescence dye. Each reaction was performed in 20 μL containing Taq DNA polymerase, the appropriate primer pair (250 nM), 0.25 mM dNTPs, 2 mM MgCl₂, SYBR I and fluorescein. After the initial denaturation at 95 °C for 3 min, 40 cycles of denaturation (95 °C, 30 sec), annealing (55 °C, 20 sec), and polymerization (72 °C, 20 sec) were performed. All assays were carried out in triplicate. The PCR products were quantified at each amplification step. The specificity of each PCR reaction was assessed by agarose gel electrophoresis and melting curve analysis determined by measuring the decline in fluorescence as temperature increased from 55 °C to 95 °C. Threshold cycles (C_T) at which the fluorescence of the PCR product SYBR I complex first exceeded the background were determined by the integrated analysis software. To compare the level of expression in different samples, the difference between the C_T of the target gene and the C_T for the tubulin standard was calculated: ΔC_T (target gene) = C_T (target) – C_T (tubulin). Relative transcript levels were calculated as: 1000 × \( 2^{-ΔC_T} \).

Results

Identification of expressed Arabidopsis GSTs

Proteolysis of a soluble protein extract from seedlings generates a complex mixture of a very large number of peptides, which can pose problems for further analysis. To reduce this complexity and to preferentially examine GSH-binding proteins such as GSTs, a GSH-affinity purification step was included prior to proteolysis. Proteins eluted from the GSH-affinity column were reduced, alkylated, and digested with trypsin. The resulting peptides were acetylated and analyzed by MS. In our initial experiments these peptides were analyzed by
MALDI-TOF-MS. Although nearly 100 peptides were detected, they could not be assigned unequivocally to specific Arabidopsis GSTs because the masses of most peptides matched the predicted masses of peptides from more than one Arabidopsis GST (data not shown). Therefore, tandem mass spectrometry (MS/MS) was used to obtain information about the amino acid sequences of peptides. This allowed for unambiguous identification of the specific GSTs from which most of these peptides were derived.

This approach was used to analyze and identify the GST proteins expressed in Arabidopsis seedlings. The recovery of proteins after GSH-affinity chromatography indicates that GSTs comprise approximately 0.1% of the soluble protein in Arabidopsis seedlings. A total of 87 peptides obtained from this affinity-purified sample were analyzed by MS/MS. Based on high probability matches obtained using the Mascot peptide identification program (http://www.matrixscience.com), 38 of these peptides were identified as being derived from one of eight Arabidopsis GSTs: AtGSTF2, AtGSTF6, AtGSTF7, AtGSTF8, AtGSTF9, AtGSTF10, AtGSTFU19, or AtGSTU20 (Table I). At least three unique peptides were detected for each of these GSTs except for AtGSTU20, which was represented by a single peptide. Another 24 peptides were identified as being derived from Arabidopsis GSTs, but similarities among the members of this protein family prevented these peptides from being unequivocally assigned to a single GST. Consequently, these peptides were excluded from further analyses. For 22 peptides, the MS/MS spectra did not give a significant match with any protein in the database (data not shown). Only one peptide (IQNGCSNVVSVDADSVVDGY) was identified that had a significant match to a protein other than an Arabidopsis GST. The function of this protein, encoded by gene At2g28540, is unknown and it does not share significant sequence homology with any other characterized gene product. These results demonstrate that this technique can be
used to identify GST proteins expressed in Arabidopsis seedlings, and that GSH-affinity chromatography provides an efficient method to reduce the complexity of protein samples, allowing for analysis focused on GSTs.

**Altered expression of GSTs in response to copper and benoxacor**

RNA expression of plant GSTs is influenced by many factors, but it is not known if expression of GST proteins parallels transcript accumulation under all these conditions. By combining MS-based analysis of peptides with differential isotopic labeling, it is possible to quantify changes in protein expression (19). In our experiments, GSH-affinity purified proteins were digested with trypsin and labeled with either acetate (control) or trideuteroacetate (treated) before being combined and analyzed by MS/MS. As the labeling reaction targets primary amino groups, the amino terminus of every peptide was acetylated. In addition, if the peptide contained a lysine, this residue was labeled on its ε-amino group. Peptides that contain only a single amino-terminal primary amine appear in the mass spectrum as a pair of peaks, i.e. a doublet separated by three mass units, with one peak from the control sample and the other from the treated sample (Fig. 1a, b). Singly-charged peptides that also contain a lysine residue appear as doublets separated by six mass units. However, most peptides derived from Arabidopsis GSTs were ionized with two protons (i.e. doubly-charged). Therefore, a lysine-containing doubly-charged peptide appears as a doublet separated by three mass units (Fig. 1c, d).

Differences in expression of specific GSTs between control and treated samples were determined by comparing the relative abundance of the monoisotopic peaks of peptides derived from each protein. This technique was used to analyze the influence of copper and benoxacor on Arabidopsis GST protein expression. Specifically, two independent experiments were carried out by combining a control sample derived from untreated Arabidopsis seedlings with an
experimental sample derived from either copper- or benoxacor-treated Arabidopsis seedlings. For example, the abundance of a peptide derived from \textit{At}GSTF7 was nearly 10 fold higher in copper-treated seedlings (Fig. 1a), whereas benoxacor had essentially no effect (Fig. 1b). In contrast, a peptide derived from \textit{At}GSTU19 was approximately 6-fold more abundant in benoxacor-treated seedlings (Fig. 1d) but increased only 2-fold after copper treatment (Fig. 1c).

With few exceptions, the peptides detected in samples from both copper- and benoxacor-treated seedlings were the same as those observed in control tissues. Therefore, GSTs that were not expressed under control conditions remained silent (or below the limit of detection of these experiments) after treatment with copper or benoxacor. The relative expression of each peptide in response to copper and benoxacor was calculated based on the monoisotopic peak intensities of each doublet (Table I). Seven of the eight expressed GSTs were represented by three or more peptides. Changes in protein expression of these seven GSTs were estimated by averaging the relative abundance of all peptides derived from each protein (Fig. 2). Individual peptides derived from the same GST generally showed very similar changes in abundance within a treatment, providing some internal validation of this approach.

Copper increased the abundance of three GSTs by at least 3-fold, with \textit{At}GSTF7 being induced approximately 10-fold. \textit{At}GSTU19 was also somewhat more abundant in copper-treated seedlings. In contrast, benoxacor strongly induced \textit{At}GSTU19, as previously documented (8), but no other GSTs were significantly more abundant after treatment with this herbicide safener. Neither copper nor benoxacor had a pronounced effect on expression of \textit{At}GSTF8 or \textit{At}GSTF10. However, the abundance of \textit{At}GSTF9 was reduced more than 50 % by both treatments. A similar reduction in abundance of \textit{At}GSTU20 was observed, although this was based on analysis of a single peptide derived from this protein (Table I).
Immunoblot analysis of *At*GSTU19 and *At*GSTF2

Proteomic analysis of Arabidopsis GSTs purified from copper- and benoxacor-treated seedlings demonstrated differential expression of several Arabidopsis GSTs including *At*GSTU19 and *At*GSTF2. To corroborate these data, immunoblot analyses were carried out using antibodies raised against recombinant forms of *At*GSTU19 and *At*GSTF2. *At*GSTU19 protein expression was induced in response to benoxacor, and to a lesser extent, copper, whereas *At*GSTF2 expression was induced by copper but was unaffected by benoxacor (Fig. 3). Immunoblot analyses of the effects of copper and benoxacor on expression of *At*GSTU19 and *At*GSTF2 produced results similar to those obtained in the proteomic analyses described above. Anti-*At*GSTF2 antibodies detected an additional protein with a slightly lower molecular weight than that of *At*GSTF2 (Fig. 3). Similar results have been obtained previously (21), but the nature of this smaller protein has not been investigated further.

Expression of Arabidopsis GST RNAs in response to copper and benoxacor

RNA blot analysis was used to compare the RNA expression of the seven abundantly expressed GSTs identified in the proteomic analyses with their protein accumulation patterns. Copper treatment increased the RNA expression of *At*GSTF2, *At*GSTF6, *At*GSTF7, and to a lesser extent, *At*GSTU19, whereas benoxacor induced the RNA expression of *At*GSTU19 (Fig. 4). These results correspond with the observed changes in abundance of these GST proteins under the same conditions. However, RNA expression of *At*GSTF10 and *At*GSTF8 was induced by copper and benoxacor, respectively (Fig. 4) while protein expression experiments showed that the abundance of *At*GSTF10 and *At*GSTF8 was not affected by either treatment (Fig. 2). In addition, neither copper nor benoxacor affected RNA expression of *At*GSTF9 (Fig. 4) even though *At*GSTF9 protein expression was down-regulated in response to these stimuli (Fig. 2).
RNA expression of At2g28540, the gene encoding the non-GST protein that was induced by copper, was also examined. Because this transcript could not be detected by blot hybridization, real-time RT-PCR was used. Relative to expression of a control tubulin RNA, the transcript of At2g28540 was approximately five-fold higher in copper-treated seedlings ($\Delta C_T$ of $-1.25 \pm 0.1$) compared to control seedlings ($\Delta C_T$ of $-3.60 \pm 0.5$).

**Discussion**

RNA expression of genes encoding GSTs is influenced by diverse abiotic and biotic factors in plants and other organisms. However, relatively little is known about the effects of these stimuli on expression of plant GST proteins. As a result, many questions remain regarding the regulation and functions of plant GSTs. To examine the expression of multiple GST proteins under various environmental conditions, we carried out a proteomic analysis of copper- and benoxacor-treated Arabidopsis seedlings.

One widely used approach to proteomics separates proteins by two-dimensional (2-D) gel electrophoresis followed by proteolysis and mass spectrometry (MS) to determine the identity of individual proteins. This method was used to identify the GSTs expressed in *Caenorhabditis elegans* (22). An alternative approach examines large numbers of proteins simultaneously by analyzing signature peptides, unique peptides that provide information about the protein from which they were derived (18). Advantages of this technique over the 2-D gel approach are that peptides are easier to separate than intact proteins, and the proteolysis step is performed only once per sample as opposed to once for each protein spot. In addition, proteins that are relatively insoluble, overlap in 2-D gels, or have a $pI$ outside the range typically used for 2-D gel separations can be analyzed more efficiently (18). The signature peptide technique is also well
suited to the use of differential isotopic labeling to quantify changes in protein abundance between control and experimental samples (19). While differential labeling with fluorescent dyes has been used to quantify proteins separated by 2-D-SDS-PAGE (23), this technique depends on quantification of the fluorescent molecules rather than the peptides themselves. In contrast, the signature peptide approach coupled with differential isotopic labeling allows for direct quantification of relative changes in protein abundance (18, 19).

The signature peptide approach was combined with differential isotopic labeling to monitor changes in GST abundance in response to copper and benoxacor. In order to focus on GSTs, and to reduce the complexity of the protein samples, GSH-affinity chromatography was included prior to trypsin digestion. Using these methods, eight GSTs were identified in Arabidopsis seedlings grown under normal conditions, and following copper- and benoxacor-treatments: \textit{AtGSTF2}, \textit{AtGSTF6}, \textit{AtGSTF7}, \textit{AtGSTF8}, \textit{AtGSTF9}, \textit{AtGSTF10}, \textit{AtGSTFU19}, and \textit{AtGSTU20}. A number of reasons could account for why only eight of the 53 GSTs encoded in the Arabidopsis genome were detected in these experiments. First, although most GSTs can be purified via GSH-affinity chromatography (24), some GSTs may not be recovered using this method. For example, recombinant \textit{AtDHAR1}, \textit{AtDHAR2}, \textit{AtDHAR3}, \textit{AtGSTL1}, and \textit{AtGSTL2} could not be purified from bacterial lysates by GSH-affinity chromatography (5). Therefore, the initial affinity purification may have excluded some GSTs from further analysis. Second, these experiments used young seedlings grown in a liquid medium. Many GSTs may only be expressed in more mature tissues, during specific developmental processes, or in response to stimuli other than those used here. Third, some GSTs may be present in quantities that are below the limit of detection for this procedure. The eight GSTs identified in this study are among the 10 Arabidopsis GSTs represented most frequently in EST collections (MatDB,
http://mips.gsf.de/proj/thal/db), suggesting they are among the most highly expressed GSTs. Therefore, this method appears to be capable of analyzing most of the abundantly expressed GSTs in Arabidopsis seedlings. Future studies will focus on characterizing GSTs expressed in specific tissues, in response to a wider variety of environmental conditions (e.g. pathogens, extreme temperatures), and during specific developmental processes.

Treatment of Arabidopsis seedlings with benoxacor and other herbicide safeners increases GST activity against the model substrate CDNB, as well as a number of chloroacetanilide herbicides (8). *AtGSTU19* was shown to be induced by safeners and was identified as the most abundant GST in safener-treated seedlings. RNA expression of *AtGSTU19* and other Arabidopsis GST genes (e.g. *AtGSTF2*, *AtGSTF7* and *AtGSTF8*) increased in response to safeners. However, in the proteomic analysis reported here, *AtGSTU19* was the only protein shown to be induced by benoxacor. This indicates that both transcriptional and translational mechanisms are involved in regulating the expression of GSTs in safener-treated Arabidopsis seedlings. The increased expression of only one Arabidopsis GST protein in response to benoxacor may contribute to the inability of this compound to safen Arabidopsis, i.e. protect it from herbicide injury. Although *AtGSTU19* is responsible for the majority of the chloroacetanilide-conjugating activity, the failure of safeners to induce other GSTs that could attenuate herbicide injury may be a critical difference between crop plants, which are protected by safeners, and Arabidopsis and other weeds.

A notable difference between the responses of Arabidopsis seedlings to copper and benoxacor is the number of GST proteins induced by these treatments. In contrast to benoxacor, which induced the expression of only *AtGSTU19*, copper induced at least four GSTs—*AtGSTF2*, *AtGSTF6*, *AtGSTF7*, and *AtGSTU19*. Overall, changes in RNA expression of GST genes in...
response to copper treatment agreed well with the changes in protein abundance, with the exception of \textit{AtGSTF10}. Although there has not been a systematic study of the effect of copper on expression of plant GSTs, independent studies have shown that RNA levels for \textit{AtGSTF2}, \textit{AtGSTF6}, and \textit{AtGSTU19} can be elevated by various copper treatments (8, 21, 25). The large number of GSTs induced by copper compared to benoxacor may reflect the toxicity of this metal to many plant processes.

Because copper can impact many different aspects of plant physiology, several mechanisms may be involved in elevating the expression of Arabidopsis GSTs. Copper can generate a number of reactive oxygen species, some of which are known to influence the activity of GST gene promoters. For example, the expression of \textit{AtGSTF8} in response to hydrogen peroxide is mediated in part by a stress-inducible \textit{ocs} (octopine synthase)-like element (26, 27). Copper may also impact GST expression as a result of the synthesis of phytochelatins, which depletes the GSH pool (28, 29). Given that the functions of many GSTs require GSH, a decrease in the availability of GSH may influence GST expression. Finally, copper may affect GST expression indirectly by inducing the biosynthesis of ethylene (30), which has been shown to induce the expression of \textit{AtGSTF2} (31) and \textit{AtGSTF6} (32). Future studies will assess if there is a common mechanism responsible for the induction of these GSTs in copper-treated seedlings.

Two GSTs identified in this study, \textit{AtGSTF6} and \textit{AtGSTF7}, are encoded by genes that share 94\% identity in the coding sequence. Because this high degree of identity extends into the promoter region, both genes appear to be similarly regulated. This is supported by RNA analyses presented here and elsewhere (8, 33). However, nucleic acid probes for \textit{AtGSTF6} and \textit{AtGSTF7} have been shown to cross-hybridize (33), raising the possibility that RNA hybridization results reflect the combined expression of both genes. Our proteomic analysis was
able to distinguish between \textit{At}GSTF6 and \textit{At}GSTF7 with at least three signature peptides identified for each (Table I). This highlights the ability of this method to distinguish between very similar gene products. Although expression of both GSTs increased in response to copper, \textit{At}GSTF7 was induced 10-fold whereas \textit{At}GSTF6 was induced only 4-fold. Future experiments will use gene-specific RNA analysis to determine whether this difference in protein expression mirrors the RNA expression of these genes in response to copper, or if there are post-transcriptional factors that affect the abundance of these closely related GSTs.

Approximately 25\% of the abundant peaks analyzed by MS/MS (22 out of 87) did not significantly match any predicted peptides from proteins in the NCBInr database. When analyzed by SDS-PAGE, the GSH-affinity fraction consisted almost entirely of proteins with molecular weights expected for GSTs (data not shown). These unidentified peptides may have originated from GSTs that had undergone some post-translational modification. However, inclusion of a number of potential modifications in the parameters for data analysis by Mascot still failed to identify the genes encoding these peptides. Difficulties in interpreting the non-random fragmentation patterns of peptides may have also contributed to the failure to match these peptides to known Arabidopsis proteins (34). The unidentified peptides could be derived from proteins that contain a GSH-binding domain or interact with GSH-binding proteins, such as GSTs. One protein was identified based on a single peptide that was not a GST. Neither the protein nor the gene, At2g28540, have been characterized. A BLAST search identified four predicted proteins in Arabidopsis and one in rice that share significant homology with At2g28540. Expression of this protein was not affected by benoxacor but was approximately 10-fold higher in copper-treated seedlings, where its RNA level was also elevated five-fold. It will be of interest to examine the nature of this protein's affinity for GSH, or for other GSH-binding
proteins such as GSTs, as well as determine if this protein is involved in adaptation to copper toxicity.

In summary, we demonstrate that the signature peptide approach is an effective strategy for examining the Arabidopsis GST family. By using other purification methods, it should be possible to examine a wide range of protein sets. For example, lectin columns can be used to purify glycosylated peptides (18), and metal-chelating columns loaded with copper or gallium can be used to purify histidine-containing peptides (35) or phosphorylated peptides (36), respectively. Eight GSTs that are expressed in Arabidopsis seedlings have been identified, and changes in expression of these GSTs in response to two different stimuli, copper and benoxacor, have been monitored. These results provide insight into the differences between RNA and protein expression patterns of specific GSTs, and emphasize the value of examining not only RNA expression, but also protein expression of GSTs to gain information about their regulation and functions. Further studies are needed to ascertain the in vivo function of each of the GSTs identified here, and to understand the roles they play both as constitutively expressed proteins and proteins induced by environmental conditions.

Acknowledgements

This research was supported in part by grants from the National Institutes of Health and the US Department of Agriculture NRI Program.
References

1. Coleman, J. O. D., Blake-Kalff, M. M. A., and Davies, T. G. E. (1997) *Trends Plant Sci.* 2, 144-151

2. Marrs, K. A. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 127-158

3. Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001) *Biochem. J.* 360, 1-16

4. Dixon, D. P., Cole, D. J., and Edwards, R. (2000) *Arch. Biochem. Biophys.* 384, 407-412

5. Dixon, D. P., Davis, B. G., and Edwards, R. (2002) *J. Biol. Chem.* 277, 30859-30869

6. Davies, J., and Caseley, J.C. (1999) *Pestic. Sci.* 55, 1043-1058

7. Irzyk, G. P., and Fuerst, E. P. (1993) *Plant Physiol.* 102, 803-810

8. DeRidder, B. P., Dixon, D. P., Beussman, D. J., Edwards, R., and Goldsbrough, P. B. (2002) *Plant Physiol.* 130, 1497-1505

9. Roxas, V. P., Smith, R. K., Allen, E. R., and Allen, R. D. (1997) *Nat. Biotechnol.* 15, 988-991

10. Cummins, I., Cole, D. J., and Edwards, R. (1999) *Plant J.* 18, 285-292

11. Roxas, V. P., Lodhi, S. A., Garrett, D. K., Mahan, J. R., and Allen, R. D. (2000) *Plant Cell Physiol.* 41, 1229-1234

12. De Vos, C. H. R., ten Bookum, W. M., Vooijs, R., Schat, H., and de Kok, L. J. (1993) *Plant Physiol. Biochem.* 31, 151-58

13. Gronwald, J. W., and Plaisance, K. L. (1998) *Plant Physiol.* 117, 877-892

14. Cummins, I., Cole, D. J., and Edwards, R. (1997) *Pestic. Biochem. Physiol.* 59, 35-49

15. Mueller, L. A., Goodman, C. D., Silady, R. A., and Walbot, V. (2000) *Plant Physiol.* 123, 1561-1570

16. Gonneau, J., Mornet, R., and Laloue, M. (1998) *Physiol. Plant.* 103, 114-124

17. Wagner, U., Edwards, R., Dixon, D. P., and Mauch, F. (2002) *Plant Mol. Biol.* 49, 515-532
18. Geng, M., Ji, J., and Regnier, F. E. (2000) *J. Chromatogr. A* **870**, 295-313

19. Regnier, F., Amini, A., Chakraborty, A., Geng, M., Ji, J., Riggs, L., Sioma, C., Wang, S., and Zhang, X. (2001) *LC GC North America*, **19**, 200-213

20. Bradford, M. (1976) *Anal. Biochem.* **72**, 248-254

21. Smith, A. P., Nourizadeh, S. D., Peer, W. A., Xu, J., Bandyopadhyay, A., Murphy, A. S., and Goldsbrough, P. B. (2003) *Plant J.* **36**, 433-442

22. Van Rossum, A. J., Brophy, P. M., Tait, A., Barrett, J., and Jefferies, J. R. (2001) *Proteomics*, **1**, 1463-1468

23. Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., and Davison, M. (2001) *Proteomics*, **1**, 377-396

24. Pascal, S., and Scalla, R. (1999) *Physiol. Plant.* **106**, 17-27

25. Mira, H., Martinez, N., and Penarrubia, L. (2002) *Planta* **214**, 939-946

26. Chen, W., Chao, G., and Singh, K. B. (1996) *Plant J.* **10**, 955-966

27. Chen, W., and Singh, K. B. (1999) *Plant J.* **19**, 667-677

28. Zenk, M. H. (1996) *Gene* **179**, 21-30

29. Mehra, R. K., and Tripathi, R. D. (2000). *Environmental pollution and plant responses*, Lewis Publishers, Boca Raton, FL

30. Yu, Y. B., and Yang, S. F. (1980) *Plant Physiol.* **66**, 281-285

31. Zhou, J., and Goldsbrough, P. B. (1993) *Plant Mol. Biol.* **22**, 517-523

32. Conklin, P. L., and Last, R. L. (1995) *Plant Physiol.* **109**, 203-212

33. Lieberherr, D., Wagner, U., Dubuis, P. H., Métraux, J. P., and Mauch, F. (2003) *Plant Cell Physiol.* **44**, 750-757

34. Tabb, D.L., Smith, L. L., Breci, L. A., Wysocki, V. H., Lin, D., and Yates, J. R. (2003) *Anal. Chem.* **75**, 1155-1163
35. Ji, J., Chakraborty, A., Geng, M., Zhang, X., Amini, A., Bina, M., and Regnier, F. (2000) J. Chromatogr. B 745, 197-210

36. Posewitz, M. C., and Tempst, P. (1999) Anal. Chem. 71, 2883-2892
Figure Legends

Fig. 1. MS spectra showing differential induction of \textit{At}GSTF7 and \textit{At}GSTU19 by copper and benoxacor. \textit{a, b} Identification of a peptide (EPFIFR) from \textit{At}GSTF7 that is induced by copper \textit{(a)} but not by benoxacor \textit{(b)}. \textit{c, d} Identification of a peptide (FANFSIESEVPK) from \textit{At}GSTU19 that is induced by copper \textit{(c)} and benoxacor \textit{(d)}. Only monoisotopic peaks were used to determine the relative abundance of the peptides. Isotope peaks due to naturally occurring $^{13}$C are labeled with asterisks. MS/MS spectra were analyzed with Mascot (http://www.matrixscience.com) to determine the identities of the peptides.

Fig. 2. Induction of Arabidopsis GSTs in response to copper and benoxacor. Relative expression of GSTs in response to copper and benoxacor were estimated by comparing peptide abundance from treated and untreated seedlings. Values are means and standard deviations of at least 3 unique peptides representing each GST. Data are displayed on a logarithmic scale. A value of 1 denotes a level of expression equal to that of the control.

Fig. 3. Immunoblot analysis of \textit{At}GSTU19 and \textit{At}GSTF2 in copper- and benoxacor-treated Arabidopsis seedlings. Seven-day-old seedlings grown in liquid medium were treated for 24 h with 50 $\mu$M CuSO$_4$ or 100 $\mu$M benoxacor. Total soluble proteins were extracted, separated (15 $\mu$g per lane) using SDS-PAGE, and transferred to nitrocellulose membranes. Blots were probed with antiserum raised against recombinant \textit{At}GSTU19 (26.8 kDa) or \textit{At}GSTF2 (24.1 kDa).
Fig. 4. RNA expression of Arabidopsis GSTs following copper and benoxacor treatment.

Seven-day-old seedlings grown in liquid medium were treated for 24 h with 50 µM CuSO₄ or 100 µM benoxacor. Total RNA was extracted, separated (10 µg per lane) on a 1.2% agarose gel, transferred to a nitrocellulose membrane, and hybridized with cDNAs encoding various Arabidopsis GSTs as indicated. Equal RNA loading was confirmed by the ethidium bromide-staining of rRNA as shown in the bottom panel.
Table I. Peptides identified in samples derived from untreated, copper-, and benoxacor-treated Arabidopsis seedlings. Relative expression is the change in abundance compared to the control. ND = No data (due to the absence of the peptide from the corresponding sample).

| GST      | m/z   | Charge | Peptide sequence                        | Relative expression |
|----------|-------|--------|-----------------------------------------|---------------------|
|          |       |        | Copper | Benoxacor | Copper | Benoxacor |
| GSTF2    | 540.3 | 2      | LAFEQIFK                               | 2.61                | 1.11    |
|          | 599.3 | 2      | VFGHPASIATR                            | 1.89                | 1.21    |
|          | 757.7 | 3      | AIMAIGMQVEDHQFDPVASK                   | 5.02                | 1.26    |
|          | 847.9 | 2      | YENCQTNLLQTDSDK                        | 3.34                | 0.93    |
|          | 959.4 | 3      | NISQYAIMAIGMQVEDHQFDPVASK              | 2.46                | 1.15    |
|          | 1005.0| 2      | SIYGLTTDEAVVAEEAK                      | 4.88                | 0.89    |
|          | 1033.2| 3      | YLAGETFTLTDLHILPAAQYLLGPTK             | 3.28                | 1.14    |
| GSTF6    | 780.4 | 2      | NVDFEFVHVLEK                           | 5.21                | 1.25    |
|          | 803.9 | 2      | AITQYIAHEFSOK                          | 3.70                | 1.07    |
|          | 987.5 | 1      | GNNULLSTGK                             | 3.06                | 1.10    |
| GSTF7    | 543.8 | 2      | GNQLVSLGSK                             | 11.75               | 1.07    |
|          | 780.4 | 1      | YLASDK                                 | 8.00                | 1.14    |
|          | 794.4 | 2      | NLDREFVHIELK                           | 10.22               | ND      |
|          | 850.4 | 1      | EPFIIFR                                | 9.67                | 1.25    |
| GSTF8    | 677.3 | 1      | LAFER                                  | 1.31                | 1.21    |
|          | 759.4 | 2      | DLQFELIPvDMR                           | 1.52                | 1.40    |
|          | 799.5 | 1      | VIDLQK                                 | 1.38                | 0.92    |
|          | 845.4 | 1      | VSEWIK                                 | 1.26                | 1.14    |
|          | 1020.5| 1      | VLATLIEK                               | 1.16                | 1.10    |
|          | 1159.0| 2      | ATTNWVLQVEGQQFDPNASK                   | 1.27                | 1.27    |
| GSTF9    | 752.3 | 2      | GVAFETIPVDMK                           | 0.47                | 0.29    |
|          | 799.9 | 2      | LGVLVDEYAEHLSK                         | 0.36                | 0.29    |
|          | 970.5 | 1      | ALVTLIEK                               | 0.49                | 0.29    |
|          | 992.8 | 3      | YLAGDFVSLADLHLPFTDLVGPQGK              | 0.62                | ND      |
| GSTF10   | 601.8 | 2      | IPVLVDGYK                              | 0.79                | 0.71    |
|          | 620.3 | 1      | YSLPV                                  | 0.94                | 0.91    |
|          | 737.8 | 2      | OPEYLAIOPFGK                           | 0.94                | 0.81    |
|          | 747.4 | 2      | VLTLYAPLFASSK                         | 1.02                | 0.84    |
|          | 831.5 | 2      | LAEVLDYEAQLSK                         | 0.84                | 0.70    |
|          | 942.5 | 1      | AVTLLEK                                | 0.89                | 0.71    |
| GSTU19   | 561.3 | 2      | VTEFVSELR                              | 1.56                | 6.24    |
|          | 663.8 | 2      | NPILPSDPLYR                            | 1.73                | 6.13    |
|          | 726.3 | 2      | FANFSIESEVPK                           | 1.84                | 5.74    |
|          | 737.9 | 2      | SPLLLQMNPPIHK                          | 1.48                | 6.12    |
|          | 858.4 | 1      | KKWAK                                  | 1.61                | 6.43    |
|          | 873.4 | 2      | SLPDPEK                                | ND                  | 5.86    |
|          | 961.5 | 1      | DFIELK                                 | 1.26                | 6.75    |
| GSTU20   | 715.9 | 2      | SPLLQSNPPIHK                           | 0.77                | 0.50    |
Fig. 1. MS spectra showing differential induction of *At*GSTF7 and *At*GSTU19 by copper and benoxacor.  

*a, b* Identification of a peptide (EPFIFR) from *At*GSTF7 that is induced by copper (*a*) but not by benoxacor (*b*).  

c, d Identification of a peptide (FANFSIESEVPK) from *At*GSTU19 that is induced by copper (*c*) and benoxacor (*d*). Only monoisotopic peaks were used to determine the relative abundance of the peptides. Isotope peaks due to naturally occurring $^{13}$C are labeled with asterisks. MS/MS spectra were analyzed with Mascot (http://www.matrixscience.com) to determine the identities of the peptides.
Fig. 2. **Induction of Arabidopsis GSTs in response to copper and benoxacor.** Relative expression of GSTs in response to copper and benoxacor was estimated by comparing peptide abundance from treated and untreated seedlings. Values are means and standard deviations of at least 3 unique peptides representing each GST. Data are displayed on a logarithmic scale. A value of 1 denotes a level of expression equal to that of the control.
Fig. 3. **Immunoblot analysis of* AtGSTU19* and* AtGSTF2* in copper- and benoxacor-treated* Arabidopsis* seedlings.** Seven-day-old seedlings grown in liquid medium were treated for 24 h with 50 µM CuSO₄ or 100 µM benoxacor. Total soluble proteins were extracted, separated using SDS-PAGE (15 µg per lane), and transferred to nitrocellulose membranes. Blots were probed with antiserum raised against recombinant* AtGSTU19* (26.8 kDa) or* AtGSTF2* (24.1 kDa).
Fig. 4. **RNA expression of Arabidopsis GSTs following copper and benoxacor treatment.**

Seven-day-old seedlings grown in liquid medium were treated for 24 h with 50 µM CuSO₄ or 100 µM benoxacor. Total RNA was extracted, separated (10 µg per lane) on a 1.2% agarose gel, transferred to a nitrocellulose membrane, and hybridized with cDNAs encoding various Arabidopsis GSTs as indicated. Equal RNA loading was confirmed by the ethidium bromide-staining of rRNA as shown in the bottom panel.
Proteomic analysis of arabidopsis glutathione S-transferases from Benoxacor- and copper-treated seedlings
Aaron P. Smith, Ben P. DeRidder, Woei-Jiun Guo, Erin H. Seeley, Fred E. Regnier and Peter B. Goldsborough

J. Biol. Chem. published online April 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402807200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts