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# Pyroxasulfone-Resistant Annual Ryegrass (*Lolium rigidum*) Has Enhanced Capacity for Glutathione Transferase-Mediated Pyroxasulfone Conjugation

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**ABSTRACT:** The herbicide pyroxasulfone was widely introduced in 2012, and cases of evolved resistance in weeds such as annual ryegrass (*Lolium rigidum* Gaud.) and tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] have started to emerge. Pyroxasulfone is detoxified by tolerant crops, and by annual ryegrass that has been recurrently selected with pyroxasulfone, in a pathway that is hypothesized to involve glutathione conjugation. In the current study, it was confirmed that pyroxasulfone is conjugated to glutathione *in vitro* by glutathione transferases (GSTs) purified from susceptible and resistant annual ryegrass populations and from a tolerant crop species, wheat. The extent of conjugation corresponded to the pyroxasulfone resistance level. Pyroxasulfone-conjugating activity was higher in radicles, roots, and seeds compared to coleoptiles or expanded leaves. Among the GSTs purified from annual ryegrass radicles and seeds, an orthologue of *Brachypodium distachyon* GSTF13 was >20-fold more abundant in the pyroxasulfone-resistant population, suggesting that this protein could be responsible for pyroxasulfone conjugation.

**KEYWORDS:** *annual ryegrass, herbicide resistance, Lolium rigidum, very-long-chain fatty acid elongase inhibitor, wheat*

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## INTRODUCTION

Annual ryegrass (*Lolium rigidum*) is the major agricultural weed of southern Australia. Its high abundance, genetic variability, and fecundity have facilitated rapid evolution of resistance to most of the herbicides commonly used for its control in cereal and broadleaf crops.[1−3] In recent years, the widespread development of resistance to heavily used in-crop (post-emergence) herbicides in no-till farming systems has led Australian growers to become very reliant on pre-emergence herbicides (i.e., those applied to the soil before the weed seedlings have emerged) to control annual ryegrass.[4] Inevitably, resistance to pre-emergence herbicides such as trifluralin, prosulfocarb, *S*-metolachlor, propyzamide, and pyroxasulfone has started to appear in field populations of annual ryegrass, with some exhibiting resistance to multiple herbicides.[5]

Pyroxasulfone (3-[[5-(difluoromethoxy)-1-methyl-3-(trifluoromethyl)pyrazole-4-yl]methylsulfonyl]-5,5-dimethyl-4*H*-1,2-oxazole) is a relatively new pre-emergence herbicide registered for use in Australia in 2012.[6] It is an inhibitor of very-long-chain fatty acid synthesis; specifically, pyroxasulfone was shown to inhibit the 3-ketoacyl-CoA synthases (KCSs) catalyzing the sequential two-carbon elongation of stearic acid (C18:0) to form the C20:0−C30:0 fatty acids in *Arabidopsis*, wheat, maize, rice, barnyard millet, and Italian ryegrass (*Lolium multiflorum*), with greater inhibition of the enzymes of the latter three species.[7,8] This differential inhibition of KCS activity among species is thought to be one reason for the selectivity of pyroxasulfone in tolerant crops such as soybean, maize, and wheat.[8] Additionally, a comparison of pyroxasulfone metabolism in wheat and annual ryegrass demonstrated that wheat has a much higher capacity for conversion of the pyroxasulfone molecule to metabolites with lower phytotoxicity.[9] In plants treated with radiolabeled pyroxasulfone, the identities of the major metabolites implied that pyroxasulfone is cleaved by conjugation of glutathione to the isoxazoline ring, although rapid subsequent metabolism and glucose conjugation meant that the initial glutathione conjugate was not detected *in vivo*.[9]

An annual ryegrass population (SLR31) exposed to low-dose recurrent selection by four applications of pyroxasulfone followed by two applications of prosulfocarb showed high-level survival (54%, compared to 5% for the parental population) after pyroxasulfone treatment, conferred by enhanced metabolism of the herbicide.[10] The same glutathione-based metabolites as identified by Tanetani et al.[9] were detected in this resistant annual ryegrass population, along with enhanced constitutive expression of a number of glutathione transferase (GST) genes.[10] The large GST superfamily of enzymes is best known for its ability to conjugate xenobiotics to glutathione, but members are also involved in antioxidant defence (acting as glutathione peroxidases or dehydroascorbate reductases), ligand binding

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Table 1. Types and Amounts of Annual Ryegrass and Wheat Tissues Used for GST Assay and Purification[b]

| | | annual ryegrass: crude extracts | | | | wheat: crude extracts | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $GST_{CDNB}$ activity | | $GST_{pyrox}$ activity | | $GST_{CDNB}$ activity | | $GST_{pyrox}$ activity | |
| tissue type | plant age | no. indiv/rep | no. reps | no. indiv/rep | no. reps | no. indiv/rep | no. reps | no. indiv/rep | no. reps |
| dry seed | n/a | 20 | 3 | 50 | 6 | n/a | n/a | n/a | n/a |
| imbibed seed | 24 h | 20 | 3 | 50 | 6 | n/a | n/a | n/a | n/a |
| germinated seed | 7 days | 20 | 3 | n/a | n/a | 8 | 3 | 8 | 3 |
| radicle | 7 days | 20 | 3 | n/a | n/a | 8 | 3 | 8 | 3 |
| coleoptile | 7 days | 20 | 3 | n/a | n/a | 8 | 3 | 8 | 3 |
| root | 7 weeks | 5 | 3 | 5 | 3 | n/a | n/a | n/a | n/a |
| shoot | 7 weeks | 5 | 3 | 5 | 3 | n/a | n/a | n/a | n/a |
| | | annual ryegrass: purified GSTs | | | | wheat: purified GSTs | | | |
| | | GST activity assay | | GST isoform abundance | | GST activity assay | | GST isoform abundance | |
| tissue type | plant age | no. indiv/rep | no. reps | no. indiv/rep | no. reps | no. indiv/rep | no. reps | no. indiv/rep | no. reps |
| coleoptile | 7 days | 400 | 1[b] | 400 | 3 | 60 | 1 | n/a | n/a |
| radicle | 7 days | n/a | n/a | n/a | n/a | 60 | 1 | n/a | n/a |
| germinated seed | 7 days | n/a | n/a | n/a | n/a | 60 | 1 | n/a | n/a |
| radicle + seed | 7 days | 400 | 1 | 400 | 3 | n/a | n/a | n/a | n/a |

[a]Imbibed seeds and 7-day-old seedlings used for enzyme assays in crude extracts (40−80% ammonium sulfate cuts) were incubated with either 0 or 37.5 nM pyroxasulfone. All other experiments were performed on tissues that had not been exposed to herbicides. [b]Due to the large amount of tissues required for GST purification and the limited number of R-P4 seeds available, only one replicate of purified GSTs was prepared and subjected to the GST assay. However, 400 annual ryegrass or 60 wheat seedlings per purification were considered representative of each population.

and transport, and stress signaling.[11] A number of studies have demonstrated the inducibility of the plant-specific phi (GSTF) and tau (GSTU) families of GSTs by xenobiotics and abiotic or biotic stress[11,12] and their ability to conjugate glutathione to herbicides such as atrazine,[13] fenoxaprop-ethyl,[14] fluorodifen,[15] metolachlor,[16] and flufenacet.[17] Higher expression of herbicide-conjugating GSTs forms the basis of crop selectivity for several herbicides and is also the means by which weed populations can evolve resistance.[13] Certain GSTs, such as GSTF1 from blackgrass (*Alopecurus myosuroides*), may also contribute to (multiple) herbicide resistance in a non-conjugating capacity by modifying the activity of other enzymes and/or stabilizing protective compounds such as flavonoids.[18]

The pyroxasulfone-conjugating activity of crude protein extracts and purified GSTs from wheat and annual ryegrass was investigated in the current study. The extent of conjugation in pyroxasulfone-resistant and -susceptible annual ryegrass populations was compared, as was conjugation in different tissues. Purified GSTs from ryegrass seedlings were subjected to proteomic analysis in an attempt to identify isoforms with higher abundance in the resistant population.

## MATERIALS AND METHODS

**Plant Material.** The well-characterized annual ryegrass population VLR1, susceptible to all herbicides,[3] was used as a pyroxasulfone-susceptible control and is hereafter referred to as "S". The pyroxasulfone-resistant population used for conjugation experiments and GST characterization was generated from a field population collected from the Western Australian wheat belt in 2010,[3] prior to the release of pyroxasulfone in Australia. Pyroxasulfone treatments and recurrent selection were performed in the same way as was done previously for population SLR31 (described in the Supporting Information Methods; see also Busi et al.[19]). Pyroxasulfone selection was performed four times to generate a population designated "R-P4", which was used in the current study. Wheat (cv. Mace) was also included as a known pyroxasulfone-metabolizing control. All plants used for biochemical experiments were grown under controlled conditions (25/15 °C day/night with a 12 h photoperiod of cool white fluorescent and light-emitting diode light at 90 $\mu$mol $m^{-2}$ $s^{-1}$). Seeds were germinated on 0.6% (w/v) agar and either used at day 7 after the start of imbibition or transplanted into potting mix (50% composted pine bark, 25% peat moss, and 25% washed river sand) and grown for a further 6 weeks with regular watering and weekly fertilization with a commercial liquid fertilizer.

**Assessment of Pyroxasulfone Resistance Levels.** Seeds from the S and R-P4 populations were imbibed on 0.6% (w/v) agar containing 0, 18.75, 37.5, 75, 150, 300, 600, or 1200 nM pyroxasulfone. After 7 days of incubation under controlled conditions, coleoptile lengths were measured as an indicator of seedling growth in the presence of pyroxasulfone. There were three replicates of 20 seeds for each pyroxasulfone concentration. Wheat was used in a similar experiment at pyroxasulfone concentrations of 0, 37.5, 150, 600, 900, 1200, 2400, and 5000 nM, with 10 seeds in each of three replicates.

**Plant Tissues Used for GST Assay and Purification.** 7-day-old annual ryegrass and wheat seedlings were separated into radicles, seeds, and coleoptiles (including the enclosed first leaf) for the assay of GST activity toward the generic substrate 1-chloro-2,4-dinitrobenzene (CDNB), hereafter referred to as $GST_{CDNB}$ activity. Pilot studies on GST activity specifically toward pyroxasulfone ($GST_{pyrox}$), however, gave inconsistent results in annual ryegrass due to difficulties in obtaining sufficient tissue for detection of activity. Therefore, radicles and seeds of 7-day-old annual ryegrass seedlings were kept together (hereafter referred to as "radicle + seed") for GST purification, and 7-week-old plants, separated into roots and shoots, were used as a more abundant source of tissue for $GST_{pyrox}$ activity assays. Table 1 summarizes the tissue types and ages of annual ryegrass and wheat used in each experiment.

**Protein Extraction.** All steps were performed at 4 °C. Plant tissues were homogenized in 3 vol of grinding buffer modified from Hristozkova et al.,[20] containing 50 mM $KH_2PO_4$ (pH 7.8), 2 mM ethylene diaminetetraacetic acid disodium salt, 2% (w/v) polyvinylpyrrolidone-40, 10% (v/v) glycerol, 0.002% (v/v) Triton X-100, 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride. When extracts were to be used for GST purification, DTT was replaced with $\beta$-mercaptoethanol. Homogenates were centrifuged at 12,000*g* for 15−30 min and the 40−80% ammonium sulfate cut of the clarified supernatant was taken.[21] The resulting protein pellet was resuspended in 20 mM Tris−HCl (pH 8.0) plus 1 mM DTT (for enzyme assays) or 100 mM Tris−HCl (pH 8.0) plus 1 mM $\beta$-mercaptoethanol (for GST purification) and desalted into a buffer of


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the same composition using Sephadex G-25 PD10 columns (GE Healthcare, Sydney, Australia). Samples were concentrated in Amicon Ultra-0.5 centrifugal filter units (Merck-Millipore, Melbourne, Australia) with a molecular weight cut-off (MWCO) of 10 kDa. Protein concentration was measured using the Bradford method[22] with Bio-Rad Dye Reagent Concentrate (Bio-Rad, Hercules, CA).

**Glutathione-Affinity Purification of GSTs.** Pierce glutathione-agarose (Thermo Scientific, Melbourne, Australia) was used to affinity-purify GSTs from ammonium sulfate-precipitated extracts of annual ryegrass and wheat seedling tissues. The affinity purification methods of Pascal and Scalla[21] and Rohman et al.[23] were used with modifications, as described in the Supporting Information Methods. Column fractions eluted with 5 mM reduced glutathione (GSH) were assayed for $GST_{CDNB}$ activity (see below); those containing activity were pooled and desalted into 20 mM Tris−HCl (pH 8.0) plus 1 mM DTT and then concentrated in 10 kDa MWCO spin filters. Samples for proteomic analysis were precipitated with 9 vol cold acetone and pelleted by centrifugation at 18,000*g* for 30 min.

**Measurement of GST Activity toward CDNB.** Analyses of $GST_{CDNB}$ activity were performed on crude ammonium sulfate-precipitated extracts or purified GSTs from annual ryegrass and wheat tissues as specified in Table 1. $GST_{CDNB}$ activity was assayed spectrophotometrically by measuring the accumulation of the CDNB−glutathione conjugate at 340 nm according to Habig et al.,[24] except that 100 mM Tris−HCl (pH 8.0) was used as the buffer. Reactions were performed at 30 °C and started with enzyme extract after recording the background rate of non-enzymatic conjugation. Negative controls containing water or boiled enzyme extract were also included and their change in absorbance subtracted from that of the test samples.

**Development of a Chromatographic Assay for the Glutathione−Pyroxasulfone Conjugate.** A chromatographic method based on that of Hatton et al.[25] was developed to measure $GST_{pyrox}$ activity by quantifying the amount of glutathione−herbicide conjugate formed. Standard solutions of the conjugate were prepared by incubating 10 mM GSH and 0.25 mg (nominal concentration, 1.3 mM) of analytical-grade pyroxasulfone (Novachem, Melbourne, Australia) at 30 °C for 24 h in a 500 μL reaction also containing 100 mM Tris−HCl (pH 8.0) and 1 mM EDTA, with vigorous shaking. The reactions were then acidified with 10 μL of 3 M HCl and clarified by centrifugation (16,000*g* for 5 min) to remove any undissolved pyroxasulfone, which has an aqueous solubility limit of 9 μM.

Control reactions lacking GSH and/or pyroxasulfone were included in order to rule out any chromatogram peaks not representing the parent compounds, the conjugate, or the cleaved 1*H*-pyrazole-4-methanesulfonic acid ring of pyroxasulfone (hereafter referred to as pyrazole sulfonic acid).

Full details of the high-performance liquid chromatography (HPLC) analysis are given in the Supporting Information Methods. Compounds were separated using a 250 × 4.6 mm Alltima C18, 5 μm particle column (Grace Davison Discovery Sciences, Deerfield, IL) and detected with a 2998 photodiode array (PDA) detector (Waters, Milford, MA). Compounds were eluted with a gradient of acetonitrile in water containing 0.1% (v/v) formic acid.

Detection and quantification of samples of pure GSH, pure pyroxasulfone, and the conjugate standard prepared as described above was at 244 nm with PDA acquisition ranging from 195 to 400 nm at a resolution of 1.2 nm. Quantification was based on peak area normalized to injection volume. Under these conditions, the retention times for the conjugate, pyrazole sulfonic acid, and parent pyroxasulfone were 11.0, 24.5, and 31.7 min, respectively. Unconjugated GSH eluted very early and was excluded from quantification.

To confirm the identity of the peaks that were only present in reactions containing both pyroxasulfone and GSH, 1 mL fractions were collected from the analytical HPLC over 40 min. Fractions corresponding to the retention times of observed peaks were analyzed by direct injection into a Waters LCT Premier XE time-of-flight mass spectrometer using an electrospray ionization (ESI) source operating in either positive or negative ionization mode (see Supporting Information Methods for full details). Peaks with masses corresponding to the glutathione conjugate, pyrazole sulfonic acid, and unreacted parent pyroxasulfone were all tentatively identified. To confirm these identities, freshly prepared conjugation reactions were separated and analyzed by in-line high-resolution LC−mass spectrometry (MS) using a Waters Alliance e2695 high-performance liquid chromatograph connected to a Waters 2998 diode array detector and Waters LCT Premier XE time-of-flight mass spectrometer with an ESI source operating in negative ionization mode. Separation was achieved using an Alltima C18 column (150 mm × 2.1 mm, 5 μm, Grace Discovery Sciences) and elution with a gradient of acetonitrile in water containing 0.1% (v/v) formic acid (see the Supporting Information Methods for full details).

In order to convert the area under the glutathione conjugate chromatogram peaks to nanomoles of GSH equivalents, a series of conjugation reactions were prepared as described above, except that the GSH concentration ranged from 0.01 to 1 mM (1.5−154 μg per reaction) and was thus limiting relative to pyroxasulfone (250 μg per reaction, nominal concentration: 1.3 mM). The area under the conjugate peak was plotted against GSH concentration to create a linear standard curve ($r^2$ = 0.998).

**Measurement of GST Activity toward Pyroxasulfone.** Once the HPLC retention time of the glutathione conjugate was known and its identity confirmed by MS, $GST_{pyrox}$ reactions with plant extracts or purified GSTs as specified in Table 1 were performed based on the method of Edwards and Cole[26] (described in full in the Supporting Information Methods). Each reaction was paired with a reaction containing boiled protein from the equivalent sample to account for the non-enzymatic conjugation of pyroxasulfone with GSH.

Positive identification of HPLC peaks in the enzyme reactions was done by comparing retention time and PDA peak spectral analyses of these peaks with those of the prepared standards, including peak purity of the glutathione conjugate, pyrazole sulfonic acid, and parent pyroxasulfone. Reaction products were quantified by comparing the area under the peaks to those of the standard curve described above.

**Proteomic Analysis of Purified GSTs.** GSTs were separated by the Laemmli system of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 15% polyacrylamide gels containing 750 mM Tris and run in 2× Tris−glycine running buffer (50 mM Tris base, 200 mM glycine, 0.2% [w/v] SDS) in order to increase the resolution between bands of a similar size.[27] Gels were stained with QC Colloidal Coomassie Brilliant Blue G250 (Bio-Rad, Sydney, Australia) and imaged on a GS900 scanning densitometer (Bio-Rad, Hercules, CA).

GSH-agarose-binding GSTs purified from radicles + seeds of the S and R-P4 annual ryegrass populations (Table 1) were identified using proteome mapping by one-dimensional LC-ESI-MS. Acetone-precipitated samples were reduced, alkylated, and digested with trypsin, and peptides were loaded onto an Acclaim PepMap 100 $C_{18}$ LC column (2 μm particle size, 150 mm) and separated with a linear gradient of water/acetonitrile/0.1% (v/v) formic acid in a Thermo UltiMate 3000 nanoflow UHPLC system coupled to a Q Exactive HF mass spectrometer (Thermo Scientific, Waltham, MA). ProteinPilot 5.0 (Sciex, Framingham, MA) was used to analyze the spectral data against a custom database (Supporting Information Table S2) of all the annotated GST sequences from *Brachypodium distachyon*[28] plus all GSTs and glutathione-binding proteins identified in the transcriptome (Busi et al. unpublished) of the pyroxasulfone- and prosulfocarb-selected annual ryegrass population SLR31-P6 generated by Busi et al.[10]

Four GST isoforms for which the number of peptides identified in the R-P4 sample was more than double of that in the S sample were investigated further by quantifying their abundance in freshly purified GST preparations from the radicles + seeds of the S and R-P4 populations using multiple reaction monitoring (MRM). Acetone-precipitated GST was solubilized in 50 mM ammonium bicarbonate, quantified using the absorbance at 280 nm, reduced with tris(2-carboxyethyl)phosphine hydrochloride, alkylated with *S*-methyl methanethiosulfonate, digested with trypsin, and quenched with


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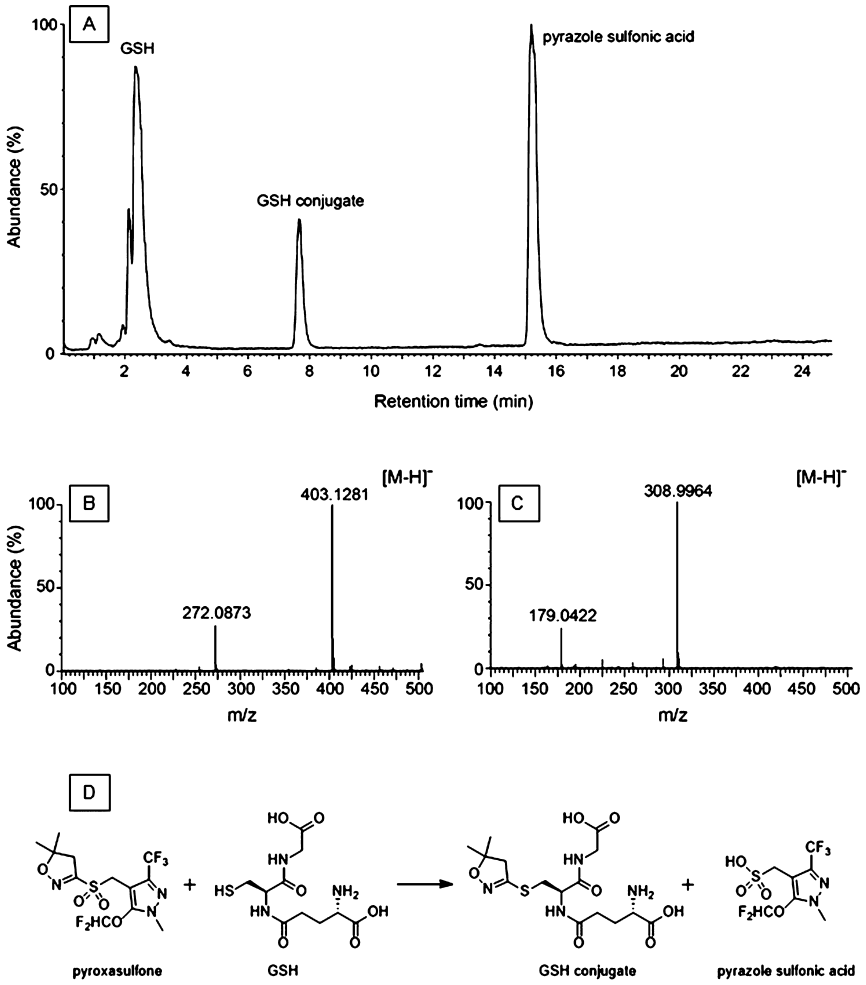


**Figure 1.** LC−MS analysis of products resulting from co-incubation of reduced glutathione (GSH) and pyroxasulfone in the absence of GST, showing the total ion chromatogram from negative ESI of the reaction mixture (A), high-resolution mass spectra of the glutathione conjugate (B) and the cleaved pyrazole sulfonic acid ring (C), and the overall reaction scheme (D).

formic acid. Peptides were separated by HPLC and then analyzed using a TSQ Altis Triple Quadrupole mass spectrometer (Thermo Scientific) in which MRM transitions were created and searched (see the Supporting Information Methods for full details of HPLC and MRM). Skyline v4.1 (MacCoss Lab Software) was used to perform peptide peak area quantitation.

**Data Analysis.** The dose−response data from seeds germinated on pyroxasulfone-containing agar were analyzed using the drc package in the statistical programme R to calculate the pyroxasulfone concentration causing a 50% decrease in coleoptile length.[29,30] Data were fitted to a three-parameter log-logistic model

$$y = \frac{d}{1 + \exp(b(\log x - \log e))}$$

where $d$ is the upper limit of relative coleoptile length (100%), $b$ is the slope of the curve, $x$ is the pyroxasulfone concentration, and $e$ is the concentration at which coleoptile elongation was inhibited by 50% (referred to as $ED_{50}$). The resistance index between populations was expressed as the ratio of $ED_{50}$ values.

Activities of $GST_{CDNB}$ and $GST_{pyrox}$ in crude plant extracts (detailed in Table 1) were analyzed by one-factor ANOVA. Differences between tissue types, populations, or herbicide treatments were compared in a pairwise manner using Welch's $t$-test in Excel.

The false discovery rates (FDRs) for identified GST proteins in the proteome mapping experiment were calculated from the reversed sequences of the custom GST database by the ProteinPilot software and were <0.1% for both the global and local FDRs. In order to be included in the analysis, proteins were required to have at least two unique peptides identified with >95% confidence. Peptide abundances from the MRM analysis (two or three peptides per protein) were normalized against the total amount of GST protein injected into the mass spectrometer (1.8−3.5 $\mu$g), and the results from each peptide averaged to give a whole-protein value for each population. Differences between populations were identified using one-factor ANOVA and Welch's $t$-test.

## RESULTS

**Pyroxasulfone Resistance Levels of Annual Ryegrass and Wheat.** The pyroxasulfone concentrations required to inhibit coleoptile elongation by 50% in seedlings germinated on agar containing this herbicide were 645 ± 95 nM for wheat, 67 ± 8 nM for annual ryegrass population R-P4, and 17 ± 2 nM for population S, as calculated from the dose−response curves in Supporting Information Figure S1. This corresponds to a resistance index of 37 for wheat and 3.8 for R-P4 when compared to the susceptible population S ($P < 0.0001$). At 37.5 nM pyroxasulfone, the concentration on which seedlings were germinated for assay of GST activity, coleoptile elongation was inhibited by 10, 40, and 80% in wheat, R-P4, and S, respectively (Supporting Information Figure S1).

***In Vitro* Cleavage and Conjugation of Pyroxasulfone.** High-resolution MS confirmed that pyroxasulfone is cleaved by conjugation of glutathione to the isoxazolinone ring.


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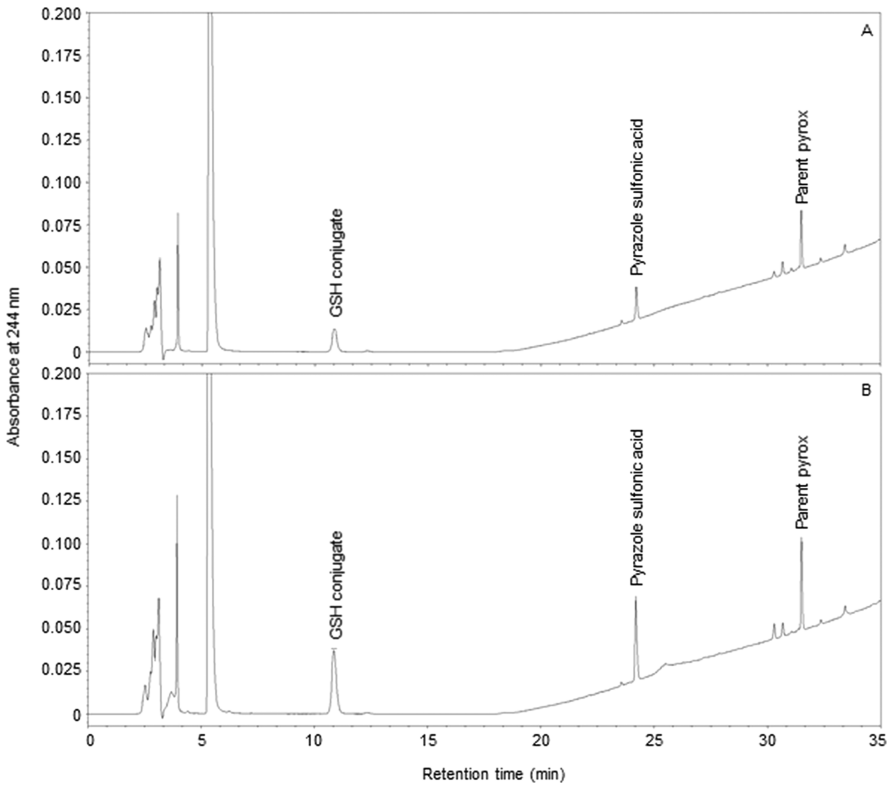


**Figure 2.** Representative HPLC chromatograms of reaction mixtures containing (A) boiled and (B) active extracts from annual ryegrass population R-P4 roots showing the separation of parent pyroxasulfone, pyrazole sulfonic acid, and the glutathione conjugate.

Glutathione, the glutathione conjugate, and pyrazole sulfonic acid eluted at 2.34, 7.67, and 15.12 min, respectively, under the conditions used for high-resolution LC−MS (Figure 1A). For the glutathione conjugate, high-resolution MS gave a molecular ion $[M - H]^-$ at $m/z$ 403.1281 ($C_{15}H_{23}N_4O_7S$ requires 403.1287), thus confirming this compound as the 5,5-dimethyl-4,5-dihydroisoxazol-3-glutathione conjugate (Figure 1B). The remaining pyrazole sulfonic acid portion of the parent pyroxasulfone molecule gave a molecular ion $[M - H]^-$ at $m/z$ 308.9964 ($C_7H_6N_2O_4SF_5$ requires 308.9968), confirming this compound as (5-(difluoromethoxy)-3-(trifluoromethyl)-1-methyl-1*H*-pyrazol-4-yl)methanesulfonic acid (Figure 1C). This suggests that the isoxazolinone-sulfonyl linkage of pyroxasulfone is cleaved and not the methylene-sulfonyl bond as previously proposed[9] since the sulfur in the glutathione conjugate must be derived from glutathione and not the parent pyroxasulfone (Figure 1D).

The glutathione conjugate and pyrazole sulfonic acid were observed by HPLC to be present in reaction mixtures containing either boiled or active enzyme, but the amounts measured were greater in the reactions containing the active enzyme (Figure 2).

**GST Activity in Annual Ryegrass.** In annual ryegrass seeds and 7-day-old seedlings incubated on either 0 or 37.5 nM pyroxasulfone, $GST_{CDNB}$ activity was not affected by the herbicide ($P > 0.05$), so the data for each herbicide treatment were pooled. The only tissues in which there was a significant difference in $GST_{CDNB}$ activity between the susceptible S and resistant R-P4 populations were the 7-day-imbibed seeds (almost threefold higher in R-P4) and the 7-week-old shoots (twofold higher in R-P4) (Table 2). Within populations, the highest activity was in the seeds and 7-day-old radicles (Table 2).

**Table 2. Activity of $GST_{CDNB}$ ($\mu$mol of Conjugate Formed $min^{-1}$ $mg^{-1}$ Protein) in Tissues from Pyroxasulfone-Susceptible (S) and -Resistant (R-P4) Annual Ryegrass Populations**[a]

| | population | |
|---|---|---|
| tissue | S | R-P4 |
| dry seed | 2.2 ± 0.3$^{ab}$ | 1.8 ± 0.1$^{b}$ |
| 24 h-imbibed seed | 2.4 ± 0.3$^{a}$ | 2.8 ± 0.2$^{a}$ |
| 7-day germ seed | 0.9 ± 0.3$^{cd}$ | 2.4 ± 0.3$^{ab}$ |
| 7-day radicle | 0.8 ± 0.2$^{cd}$ | 1.4 ± 0.5$^{abcd}$ |
| 7-day coleoptile | 0.4 ± 0.0$^{c}$ | 0.3 ± 0.1$^{cd}$ |
| 7-week root | 0.3 ± 0.0$^{c}$ | 0.4 ± 0.1$^{cd}$ |
| 7-week shoot | 0.1 ± 0.0$^{d}$ | 0.2 ± 0.0$^{c}$ |

[a]Seedlings germinated on agar for 7 days were separated into seeds, radicles, and coleoptile tissues, while 7-week-old plants were separated into root and shoot tissues. Values are means ± SE ($n = 6$ for seeds and seedling tissues; $n = 3$ for mature tissues). Different letters denote a significant ($P < 0.05$) difference between samples both within and between populations according to Welch's $t$-test.

$GST_{pyrox}$ activity in R-P4 seeds and roots was significantly (3- to 17-fold) higher than in the corresponding S samples, but activity was low in the shoots of both the S and R-P4 populations (Figure 3).

GSTs purified from radicles + seeds or coleoptiles of 7-d-old annual ryegrass seedlings were capable of conjugating GSH with pyroxasulfone but to different extents in the different populations. $GST_{pyrox}$ activity recovered from R-P4 radicles + seeds was almost 2-fold higher than that from the equivalent S tissue, while activity recovered from R-P4 coleoptiles was 9-fold higher than in the S coleoptiles (Table 3). Recovered $GST_{CDNB}$ activity was also higher in GSTs purified from the


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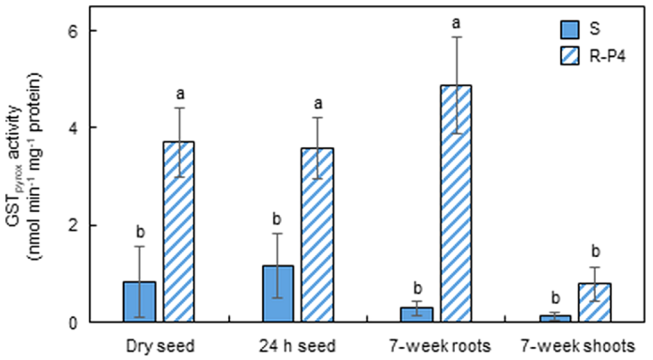


**Figure 3.** Activity of $GST_{pyrox}$ in pyroxasulfone-susceptible (S) and -resistant (R-P4) annual ryegrass populations. Soluble protein extracts from dry seeds, seeds imbibed with herbicide-free agar for 24 h, and the separated roots and shoots of 7-week-old plants were assayed. Values are means ± SE of six (seeds) or three (roots and shoots) replicates; different letters above bars denote a significant ($P < 0.05$) difference between means according to Welch's *t*-test.

**Table 3. Total $GST_{pyrox}$ and $GST_{CDNB}$ Activity Purified from 7-day-old Pyroxasulfone-Susceptible (S) and -Resistant Annual Ryegrass (R-P4) and Wheat Seedling Tissues Using GSH-Affinity Chromatography, Normalized to 1 mg of Crude Protein for Each Extract[a]**

| seedling tissue | $GST_{pyrox}$ activity (nmol $min^{-1}$) | $GST_{CDNB}$ activity ($\mu$mol $min^{-1}$) |
|---|---|---|
| S radicle + seed | 1.42 | 0.14 |
| S coleoptile | 0.13 | 0.07 |
| R-P4 radicle + seed | 2.55 | 0.48 |
| R-P4 coleoptile | 1.20 | 0.11 |
| wheat radicle | 19.85 | 1.07 |
| wheat seed | 3.22 | 0.34 |
| wheat coleoptile | 4.20 | 0.17 |

[a]Each sample represents tissues from 400 (ryegrass) or 60 (wheat) seedlings.

radicles + seeds of R-P4 compared to S (3-fold), but there was less difference between populations in the coleoptiles (1.6-fold) (Table 3).

**GST Activity in Wheat.** In crude extracts from the seeds, radicles, and coleoptiles of 7-day-old wheat seedlings incubated in the absence of pyroxasulfone, $GST_{CDNB}$ activity was the highest in the radicles, while there was no significant difference between tissues in terms of $GST_{pyrox}$ activity ($P > 0.05$) (Figure 4). Germination on 37.5 nM pyroxasulfone had no significant effect on $GST_{CDNB}$ activity in 7-day-old wheat seedlings, but $GST_{pyrox}$ activity in the radicles was ≥4-fold higher than in the pyroxasulfone-treated seeds and coleoptiles (Figure 4).

As was the case in the crude wheat extracts, the highest activity of affinity-purified $GST_{CDNB}$ and $GST_{pyrox}$ was recovered from the radicles (3- to 6-fold higher than from the seeds and coleoptiles: Table 3). On average, $GST_{pyrox}$ activity recovered from wheat was 10-fold higher than the activity recovered from annual ryegrass, but $GST_{CDNB}$ activity was only 4-fold higher (Table 3).

**Proteomic Analysis of GSH Affinity-Purified GST.** GST purified from the separated tissues of 7-day-old annual ryegrass and wheat seedlings behaved similarly on SDS−PAGE, with each sample showing a major band at 22.9 kDa (Supporting Information Figure S2; Figure 5 inset). The initial proteome

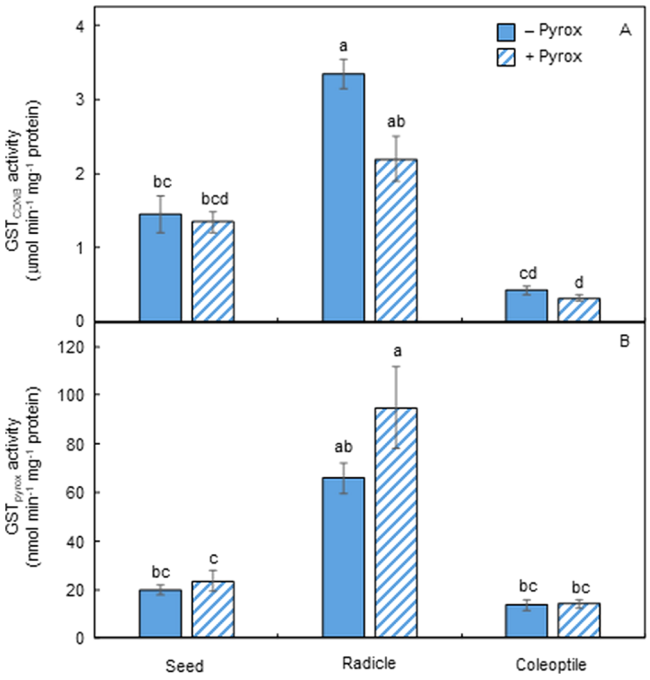


**Figure 4.** Activity of $GST_{CDNB}$ (A) and $GST_{pyrox}$ (B) in soluble protein extracts of seeds, radicles, and coleoptiles of wheat seedlings germinated on agar in the presence (+pyrox) or absence (−pyrox) of 37.5 nM pyroxasulfone and harvested at day 7 after the start of imbibition. Values are means ± SE of three replicates; different letters above bars denote a significant ($P < 0.05$) difference between means according to Welch's *t*-test.

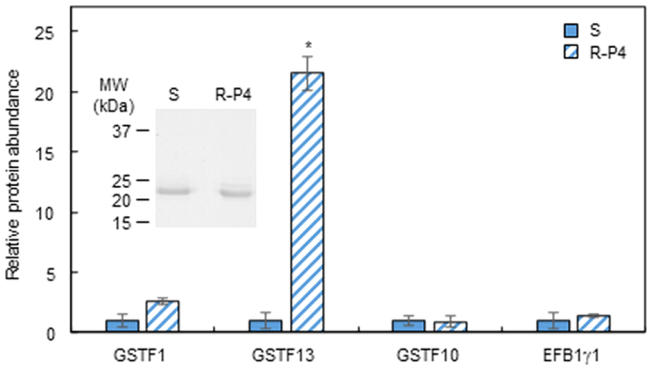


**Figure 5.** MRM-based relative quantification of four GST isoforms in affinity-purified GST from radicles + seeds of 7-day-old pyroxasulfone-susceptible (S) and -resistant (R-P4) annual ryegrass seedlings. Protein abundances were adjusted to 1 in each S sample in order to easily visualize fold-differences between S and R-P4. Values are means ± SE of three replicates; an asterisk denotes a significant difference between populations according to Welch's *t*-test ($P < 0.05$). Inset: Coomassie-stained SDS−PAGE of GSTs purified from the radicles + seeds of both annual ryegrass populations.

mapping of GST from annual ryegrass radicles + seeds resulted in the identification of 11 proteins common to both the S and R-P4 populations and another 16 proteins unique to one population (8 each) (Supporting Information Table S3). Using the number of peptides identified per protein (normalized to total $GST_{CDNB}$ activity in each preparation) as a rough estimation, proteins with sequences matching *B. distachyon* GSTF10, GSTF13, and elongation factor 1B γ1 (EF1Bγ1: a member of the GST superfamily) were more abundant in


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population R-P4 (Supporting Information Table S3). To accurately quantify these proteins as well as an orthologue of the GSTF1 conferring multiple herbicide resistance in blackgrass[18] (accession no. P6_c56551_g1 in Supporting Information Table S3), three separate preparations of GST from the radicles + seeds of both S and R-P4 were subjected to MRM analysis. Of the four proteins quantified, only GSTF13 was significantly different ($P < 0.05$) between populations, having a higher abundance of >20-fold in population R-P4 (Figure 5).

## DISCUSSION

Using an *in vitro* enzyme assay, this study has demonstrated that GSH is conjugated to pyroxasulfone in both wheat and ryegrass as predicted by Tanetani et al.[9] and that this occurs in seeds and young seedlings as well as in older plants. The conjugation reaction was catalyzed by GST purified from seedling tissues. Furthermore, the absence of substrates and/or other enzymes in the *in vitro* system prevented previously described side reactions such as liberation of glycine and glutamate from the glutathione moiety, and deamination and glucosylation of the cysteine residue, allowing unambiguous identification of the original glutathione conjugate that could not be detected in previous *in vivo* experiments.[9,10] In the conjugation reactions performed in this study, the site at which pyroxasulfone was cleaved by its reaction with GSH was confirmed by MS to be at the isoxazolinone−sulfonyl linkage.

In both annual ryegrass and wheat, the highest pyroxasulfone-conjugating activity was localized to the radicle/root and seed tissue rather than the shoot material. This is similar to the situation in maize seedlings, where the roots were not only more tolerant to the pre-emergence herbicide metolachlor than the shoots but had higher $GST_{CDNB}$ and metolachlor-conjugating GST activity.[31] The natural substrate(s) of $GST_{pyrox}$ is unknown, but the localization of this activity in the roots and seeds is optimal for detoxification of pyroxasulfone by just-germinated seedlings in soil treated with this herbicide. Although a less-sensitive target enzyme (3-ketoacyl-CoA synthase) could also contribute to the pyroxasulfone tolerance of wheat,[8] the higher pyroxasulfone-conjugating capacity of constitutive GSTs purified from wheat compared to annual ryegrass (on average, 7-fold higher than R-P4, 18-fold higher than S: Table 3) was of a similar magnitude to the higher resistance level of wheat when germinated on pyroxasulfone (10-fold more resistant than R-P4, 38-fold more resistant than S: Supporting Information Figure S1). A 3-fold higher *in vivo* production of pyroxasulfone metabolites in older wheat versus ryegrass plants was also observed in a previous study.[9] In turn, the resistance level of ryegrass population R-P4 compared to S (4-fold higher) was similar to the difference in $GST_{pyrox}$ activity in crude preparations from seeds and 7-week-old roots and shoots (average 8-fold higher in R-P4) and in purified GST from 7-day-old seedlings (average 6-fold higher). A potential candidate for the pyroxasulfone-conjugating enzyme in annual ryegrass is the orthologue of *B. distachyon* GSTF13 (annotated as GST4 in Genbank [XP_003568940.1] and glutathione transferase in UniProt [I1HLV3]), which was 20-fold more abundant in population R-P4. There is no information on the endogenous function or xenobiotic-conjugating capacity of BdGSTF13 or its closest BLAST matches (GSTF2 from wheat and barley), so confirmation of the pyroxasulfone-conjugating activity of the ryegrass orthologue, and its role in resistance, will be required *via in vitro* assay of the heterologously expressed protein and characterization of the pyroxasulfone resistance level of transgenic plants overexpressing *GSTF13*.

Direct conjugation of herbicides with GSH is an important factor in non-target-site resistance, but there is increasing evidence that GSTs can also contribute to resistance in ways that are unrelated to herbicide detoxification. Cummins et al.[18] demonstrated that upregulation of blackgrass GSTF1, an enzyme with low herbicide-conjugating ability, is associated with resistance to multiple herbicides, potentially by promoting and stabilizing the accumulation of protective flavonoids (although this has not yet been shown experimentally). A slightly (1.2-fold) higher constitutive $GST_{CDNB}$ activity in two multiple-resistant populations of wild oat (*Avena fatua*) lacking target-site mutations was not accompanied by enhanced herbicide conjugation to GSH, and it was hypothesized that repeated herbicide application had resulted in a global upregulation of genes contributing to abiotic stress tolerance.[32] In the current study on GST in annual ryegrass tissues, the overall average $GST_{pyrox}$ activity in extracts of seeds, roots, and shoots of the resistant population R-P4 was >5-fold higher ($P < 0.001$) than that of susceptible population S, while average $GST_{CDNB}$ activity was not significantly different (Table 1 and Figure 3), implying that the higher capacity for pyroxasulfone conjugation in population R-P4 is the major cause of resistance. This conclusion is supported by the fact that the abundance of the ryegrass orthologue of *A. myosuroides* GSTF1 was not significantly different between S and R-P4 (Figure 5).

In summary, the GST-catalyzed conjugation of pyroxasulfone with glutathione has been confirmed using an *in vitro* system and demonstrated to be more rapid in plants with higher resistance to pyroxasulfone. In terms of resistance management, the fact that survival of annual ryegrass had plateaued at ∼50% after three to four rounds of laboratory-based selection with pyroxasulfone (Supporting Information Table S1) indicates that the capacity for upregulation of $GST_{pyrox}$ activity in this weed species may be limited. To exploit this limitation in the field, the relative efficacy of strategic mixtures of pyroxasulfone with other herbicides needs to be investigated. For example, trifluralin is not metabolized by GST action,[33,34] so resistance to a mixture of pyroxasulfone and trifluralin would require deployment of two separate detoxification systems. Alternatively, an attempt to overwhelm the GST system by mixing pyroxasulfone with another GST-detoxified herbicide (potentially prosulfocarb or triallate) may be more effective. The current study highlights the need for detailed knowledge of herbicide detoxification pathways in order to limit the selection and evolution of resistance in weeds.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c07458.

Supplementary Methods; Generation of pyroxasulfone-resistant populations by recurrent selection; GST- and glutathione-binding protein sequences used for protein identification; proteins identified in the proteome mapping of purified annual ryegrass GSTs; pyroxasulfone dose−response curves for annual ryegrass and wheat; and polyacrylamide gel electrophoresis of purified GSTs (PDF)



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