Title: Metabolic pathways for S-metolachlor detoxification differ between tolerant corn and multiple-resistant waterhemp

Short title: S-metolachlor detoxication in resistant waterhemp

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Summary statement
Cytochrome P450 monooxygenases detoxify S-metolachlor in resistant waterhemp (Amaranthus tuberculatus) while glutathione S-transferases metabolize S-metolachlor in tolerant corn, highlighting the innate ability of waterhemp to adapt and evolve complex and contrasting resistance mechanisms.
Abstract

Herbicide resistance in weeds can be conferred by target-site and/or non-target-site mechanisms, such as rapid metabolic detoxification. Resistance to the very-long-chain fatty acid (VLCFA)-inhibiting herbicide, S-metolachlor, in multiple-herbicide resistant populations (CHR and SIR) of waterhemp (Amaranthus tuberculatus) is conferred by rapid metabolism compared with sensitive populations. However, enzymatic pathways for S-metolachlor metabolism in waterhemp are unknown. Enzyme assays using S-metolachlor were developed to determine specific activities of glutathione S-transferases (GSTs) and cytochrome P450 monooxygenases (P450s) from CHR and SIR seedlings to compare with tolerant corn and sensitive waterhemp (WUS). GST activities were greater (~2-fold) in CHR and SIR compared to WUS, but much less than corn. In contrast, P450s in microsomal extracts from CHR and SIR formed O-demethylated S-metolachlor, and their NADPH-dependent specific activities were greater (>20-fold) than corn or WUS. Metabolite profiles of S-metolachlor generated via untargeted and targeted liquid chromatography-mass spectrometry from CHR and SIR differed from WUS, with greater relative abundances of O-demethylated S-metolachlor and O-demethylated S-metolachlor-glutathione conjugates formed by CHR and SIR. In summary, our results demonstrate S-metolachlor metabolism in resistant waterhemp involves Phase I and Phase II metabolic activities acting in concert, but the initial O-demethylation reaction confers resistance.

Keywords: Amaranthus, cytochrome P450, xenobiotic detoxification, glutathione S-transferase, herbicide metabolism, weed resistance
Introduction

Plants are universally exposed to natural and synthetic toxins, including pollutants, heavy metals, allelochemicals and pesticides. As a result, plants must mount specific and coordinated defense mechanisms to ensure survival. An example defense mechanism is the ability to metabolize synthetic, organic compounds or ‘xenobiotics’ (Sandermann 1992), including herbicides. Plants detoxify herbicides via complex, multi-step processes that exhibit extraordinary diversity among cultivated and weedy species (Kreuz et al., 1996; Nandula et al. 2019) and generally require the sequential activities of several metabolic enzymes. Herbicide detoxification typically occurs in four distinct phases (I-IV) characterized by their unique properties, enzymes and reactions catalyzed (van Eerd et al. 2003; Riechers et al. 2010; Zhang and Yang 2021). Phase I reactions commonly involve cytochrome P450 monooxygenase (P450) enzymes, which can either detoxify or bioactivate herbicides (Nandula et al. 2019; Zhang and Yang 2021). P450 enzymes are encoded by large multigene families, with over 240 genes encoding P450s in Arabidopsis thaliana (Paquette et al. 2009; Mizutani and Ohta 2010; Schuler 2015; Dimaano and Iwakami 2020). Oxidative reactions catalyzed by P450s include ring and aryl hydroxylation, N- and O-dealkylation, and hydrolysis of diverse endogenous and xenobiotic substrates (Mansuy 1998; Siminszky 2006; Hamberger and Bak 2013; Li et al. 2020).

Phase II reactions are catalyzed by glutathione S-transferases (GSTs) or uridine diphosphate-dependent glycosyltransferases (UGTs) that conjugate reduced glutathione (GSH) or sugars, respectively, to herbicide substrates (Shimabukuro et al. 1978; Kreuz et al. 1996; Cummins et al. 2011). GST proteins in plants are multi-functional and diverse in the reactions they catalyze (Csiszár et al. 2019). GSTs are encoded by more than 50 genes in Arabidopsis thaliana (Estévez and Hernandez 2020), which are comprised of several subclasses with varying substrate specificities (Mashiyama et al. 2014). GST subunits can also form homo- and heterodimers that further affect substrate specificity (Dixon et al. 2002). Phase III detoxification processes involve transport and compartmentation of herbicide metabolites in the vacuole or cell wall (Sandermann 1992), and are mediated by ATP-binding cassette transporters (Kreuz et al. 1996; Schulz and Kolukisaoglu 2006; Yazaki 2006). Phase IV reactions follow cellular compartmentation; for example, GSH-herbicide conjugates are catabolized by peptidases to
recycle glutamate and glycine from the tripeptide GSH (Lamoureux and Rusness 1981; 1993; Wolf et al. 1996; Martin et al. 2007; Riechers et al. 2010).

Metabolic herbicide resistance severely threatens modern weed management practices and crop production (Yu and Powles 2014; Bagavathiannan and Davis 2018). Metabolic resistance in weedy, dioecious amaranths, such as waterhemp (Amaranthus tuberculatus Moq. Sauer), is common and typically involves detoxification reactions catalyzed by either GSTs or P450s (Ma et al. 2013; Nandula et al. 2019). Waterhemp is a pernicious, small-seeded, C4 dicot distributed throughout the United States (Sauer 1955; Steckel 2007) that is capable of generating thousands of seeds per female plant. Discontinuous germination allows waterhemp seedlings to emerge and compete throughout the growing season (Hartzler et al. 1999; Buhler and Hartzler 2001; Steckel et al. 2003; Steckel 2007), which can significantly reduce corn (Zea mays L.) and soybean (Glycine max (L.) Merr) grain yield (Hager et al. 2002; Steckel and Sprague 2004). Additionally, waterhemp is dioecious (Murray 1940; Sauer 1955) and obligate outcrossing results in high intraspecific diversity and genetic variability compared with self-pollinated species (Ward et al. 2013; Muyle et al. 2020). Widespread perpetuation of herbicide resistance in weeds is predictable as an adaptive trait in response to strong and persistent selection pressures (Bagavathiannan and Davis 2018). Unsurprisingly, waterhemp as a species has evolved resistance to myriad herbicides representing seven sites-of-action (SoA) groups (Heap 2021; Tranel, 2021), most recently to inhibitors of very-long-chain fatty acid (VLCFA) biosynthesis (Heap 2021). Multiple resistance to herbicides from up to six SoA groups has been reported in several field populations (Shergill et al. 2018; Evans et al. 2019; Strom et al. 2019).

The soil-applied, VLCFA-inhibiting herbicide S-metolachlor is effective for controlling annual grass and small-seeded dicot weeds (Fuerst 1987), including waterhemp and Palmer amaranth (Amaranthus palmeri S. Watson), in agronomic systems including corn, grain sorghum (Sorghum bicolor L. Moench ssp. bicolor), and soybean. VLCFAs with acyl chains >18 carbons are essential for the formation of cell membranes, cuticle waxes and lipids (Bach and Faure 2010) and are formed by the membrane-bound VLCFA-elongase complex (Fehling and Mukherjee 1991; Haslam and Kunst 2013). The VLCFA-elongase complex consists of four enzymes (Haslam and Kunst 2013). S-metolachlor competitively inhibits the first subunit, VLCFA synthase, resulting in
VLCFA depletion (Böger 2003; Trenkamp et al. 2004). S-metolachlor is a resolved isomer of racemic metolachlor, which normally exists in four stereoisomers (Muller et al. 2001), but S-isomers provide the majority of herbicidal activity (Moser et al. 1983).

Natural tolerance to VLCFA-inhibiting herbicides is due to Phase II reactions mediated by GSTs (Fuerst 1987; Owen 2000), and is based on nucleophilic displacement of a leaving group (typically a chlorine atom) by the thiolate anion of GSH. In general, tolerant crops or weeds have higher GST activity, GSH content, or both compared with sensitive species (Breaux 1987; Hatton et al. 1996; van Eerd et al. 2003). Mechanistic research of VLCFA-inhibitor-resistant dicot weeds is limited, but experiments with the GST inhibitor, 4-chloro-nitrobenzofurazon, suggested GSTs are involved in conferring S-metolachlor resistance in waterhemp (Strom et al. 2020) and Palmer amaranth (Brabham et al. 2019). Previous studies demonstrated microsomal fractions containing P450s from crop species oxidize metolachlor as a substrate (Moreland et al. 1990; 1993; 1995). Additionally, several oxidative metabolites have been identified in corn (Xie et al. 2010). P450-mediated herbicide metabolism has been postulated as a resistance mechanism to the VLCFA inhibitors pyroxasulfone and S-metolachlor in Lolium (Tardiff and Powles 1999; Busi 2014; Busi et al. 2017) and S-metolachlor in waterhemp (Strom et al. 2020).

We recently reported two multiple herbicide-resistant waterhemp populations from Illinois, USA (CHR from Champaign County; SIR from McLean County) that are resistant to VLCFA inhibitors (Strom et al. 2019). We then determined that the mechanism of S-metolachlor resistance was enhanced herbicide metabolism compared with sensitive waterhemp by utilizing HPLC and radiolabeled herbicide (Strom et al. 2020). However, our previous research did not unequivocally determine the enzyme families responsible for enhanced S-metolachlor metabolism. Based on our previous results, we hypothesized the pathways for S-metolachlor metabolism in CHR and SIR are different than the tolerance mechanism in corn, and that S-metolachlor detoxification involves both P450s and GSTs (Strom et al. 2020). To directly test this hypothesis, we first developed methods for waterhemp seedlings to study the activity of each enzyme with S-metolachlor as substrate. The objectives of our present study are to: (1) determine the relative contribution of GSTs and P450s in enhanced S-metolachlor metabolism
in resistant waterhemp, and (2) investigate initial metabolites formed during detoxification of S-metolachlor and deduce metabolic pathways in CHR and SIR.

Results

Resistant waterhemp and corn possess greater GST activity with S-metolachlor than sensitive waterhemp

GST activity of three waterhemp populations (CHR, SIR, WUS) and corn was measured using radiolabeled S-metolachlor (Fig. 1A) as substrate. CHR and SIR are resistant to S-metolachlor via enhanced metabolism, WUS is sensitive to S-metolachlor (Strom et al. 2019), and corn is naturally tolerant via rapid GST-mediated metabolism (Fuerst 1987). Specific GST activities from each population varied and the population effect was significant (P < 0.0001; Table 1). Corn had 5-fold more activity than WUS and 2.6- to 3.0-fold more activity than SIR and CHR, respectively (Table 1). Specific GST activities from CHR and SIR were similar, but were 1.7- to 2.0-fold greater than WUS, respectively. These results demonstrate that CHR and SIR have elevated GST activity with S-metolachlor compared with WUS. Although our previous research demonstrated CHR and SIR metabolize S-metolachlor as rapidly as corn (Strom et al. 2020), these results indicate CHR and SIR do not possess equivalent GST activity as tolerant corn (Table 1), thus suggesting additional enzyme(s) are involved.

Microsomes prepared from resistant waterhemp oxidize S-metolachlor

Oxidative activities of microsomes prepared from the waterhemp populations and corn were analyzed using radiolabeled S-metolachlor as substrate. Extracts from each waterhemp population formed a single major metabolite, which was tentatively identified as O-demethylated S-metolachlor by co-chromatography (Rf 16.0 min) with an authentic standard (Fig. 1B; 2A-C). The identity of this metabolite was further verified by liquid chromatography-mass spectrometry (LC-MS). The metabolite formed by CHR and SIR microsomes had the same mass-to-charge ratio (m/z) as the authentic standard (m/z 270) under positive ionization mode.
Additionally, the fragmentation pattern of the metabolite formed by CHR and SIR microsomes was the same as the standard, including major fragments with \( m/z \) of 176, 184 and 252.

\( O \)-demethylated S-metolachlor was more abundant in assays using CHR and SIR microsomes than from corn or WUS (Fig. 2A-D). Specific activities of CHR and SIR microsomes were much greater than corn or WUS (\( P < 0.0001 \); Table 2). CHR had 21- to 30-fold higher specific P450 activity than WUS or corn, respectively. SIR had 28- to 39-fold higher specific activity than WUS or corn, respectively, and 1.3-fold greater specific activity than CHR. Product formation was active enzyme and NADPH dependent (Fig. 2D-F), corresponding to the requirement of P450 enzymes to exist in a reduced state for catalytic activity (Werk-Reichhart and Feyereisen 2000; Siminszky 2006). These results demonstrate that CHR and SIR microsomes have a greater ability to oxidize S-metolachlor than the WUS population and corn, thus contributing to the rapid metabolism of S-metolachlor previously reported in resistant waterhemp (Strom et al. 2020).

**Untargeted LC-MS reveals differences between resistant and sensitive waterhemp**

Untargeted LC-MS was conducted to compare the metabolite profiles of CHR, SIR, and WUS following treatment with unlabeled S-metolachlor at three time points. Principal component analysis (PCA) of metabolite features demonstrated the metabolite profiles of resistant waterhemp populations, CHR and SIR, were different compared to the sensitive population, WUS (Fig. 3A), with population (PC1) and herbicide treatment (PC2) explaining approximately 35% and 10% of the variability in the dataset, respectively. LC-MS revealed S-metolachlor and GSH-S-metolachlor conjugates (and its catabolites) were more characterizing for WUS, whereas \( O \)-demethylated S-metolachlor and its respective GSH-derived conjugates and catabolites were characterizing for CHR and SIR (Fig. 3B). Identities of S-metolachlor and its metabolites were confirmed via cross referencing with the accurate mass and peak retention times of the analytical standards, which included S-metolachlor, \( O \)-demethylated S-metolachlor and *in vitro* synthesized GSH, CYS-GLY, and CYS conjugates of S-metolachlor and \( O \)-demethylated S-metolachlor. These results support the *in vitro* GST and P450 assays results and indicate oxidative metabolism and GST-mediated conjugation reactions involving S-metolachlor occur in...
resistant populations. These untargeted experiments guided subsequent targeted LC-MS experiments to quantify the relative abundance of each metabolite in S-metolachlor-treated seedlings of each waterhemp population.

**The abundance of S-metolachlor-derived metabolites differs in resistant and sensitive waterhemp seedlings**

Targeted high-resolution LC-MS was conducted to investigate and quantify S-metolachlor metabolites formed in CHR, SIR, and WUS. S-metolachlor, O-demethylated S-metolachlor and their respective GSH, CYS-GLY and γ-GLU-CYS dipeptide, and CYS conjugates were included as reference standards and relative abundances were determined. At each time point, the relative abundance of S-metolachlor was less in CHR and SIR than WUS (Fig. 4). In contrast, the GSH, CYS-GLY, γ-GLU-CYS, and CYS conjugates of S-metolachlor were similar or in greater relative abundance in WUS than in CHR or SIR during the time course (Fig. 4). However, CHR and SIR formed more O-demethylated S-metolachlor initially than WUS, and the GSH, CYS-GLY, and CYS conjugates of O-demethylated S-metolachlor were also more abundant in CHR and SIR than WUS (Fig. 5). The γ-GLU-CYS dipeptide conjugate of O-demethylated S-metolachlor was not detected (Supplementary Fig. S1). These results imply that P450 and GST activities are involved in S-metolachlor detoxification reactions in resistant waterhemp.

**Putative glucose conjugates are more abundant in resistant waterhemp than sensitive waterhemp**

Following Phase I metabolic reactions (e.g., O-dealkylation and hydroxylation), oxidized herbicides are predisposed to Phase II sugar conjugation, such as glucose, catalyzed by UDP-dependent glucosyl transferases (Werk-Reichhart and Feyereisen 2000; Osmani et al. 2009; Riechers et al. 2010). Plant extracts from CHR, SIR, and WUS containing radiolabeled S-metolachlor and its metabolites were subsequently hydrolyzed with β-glucosidase to determine if glucose conjugates were present (Lamoureux et al. 1991; Edwards 1994) by comparing β-glucosidase-treated and nontreated metabolite profiles via HPLC. One polar metabolite (Rf 3.3 min) was detected in all populations, but was more abundant in CHR and SIR than WUS (Fig. 6).
In the absence of β-glucosidase, this polar metabolite accounted for 20 and 24% of total radioactivity detected in CHR and SIR extracts, respectively, but only 6% in WUS (Fig. 6A,C,E). The abundance of this putative glucose conjugate decreased by 5, 7, and 1% for CHR, SIR, and WUS, respectively, following treatment with β-glucosidase (Fig. 6B,D,F). β-glucosidase hydrolysis concurrently increased the abundance of O-demethylated S-metolachlor in each extract (R; 19.4 min; Fig. 6), indicating that this metabolite was the substrate for glycosidation.

Discussion

Results from our current research expand upon previous findings that resistance to S-metolachlor in waterhemp is due to enhanced metabolism (Strom et al. 2020) and demonstrate S-metolachlor detoxification reactions are catalyzed by both major plant detoxification enzyme families (P450s and GSTs), as well as subsequent sugar conjugation reactions (likely by UGTs; Fig. 7). Enzyme assays with S-metolachlor as substrate indicated CHR and SIR have greater GST activity compared to WUS, but less than corn, which is naturally tolerant via this mechanism (Fuerst 1987). Further investigation of P450 activity revealed that CHR and SIR microsomes possess a greater ability to oxidize S-metolachlor through O-demethylation than WUS or corn, and this oxidation reaction appears to be the predominant resistance mechanism. P450 and GST activities likely contribute to resistance in CHR and SIR and both enzymes metabolize S-metolachlor.

Investigations into VLCFA-inhibitor resistance overall are limited and have mostly focused on monocot species. Our results represent the benchmark report of enzymatic processes underlying S-metolachlor resistance in a dicot weed. Previously, the involvement of GSTs in S-metolachlor resistance was postulated in Palmer amaranth (Brabham et al. 2019) and waterhemp (Strom et al. 2020) based on results with a chemical GST inhibitor. GSH conjugation was also proposed as a mechanism for VLCFA-inhibitor resistance in Lolium spp. (Busi et al. 2018; Dücker et al. 2019). The vast majority of research on GST activities and metabolism of VLCFA-inhibiting herbicides, however, has focused on crop selectivity mechanisms (Owen 2000; Riechers et al. 2010; Cummins et al. 2011). In general, certain crop and weed species are endowed with natural tolerance to VLCFA-inhibiting herbicides through rapid conjugation to
GSH or homoglutathione (hGSH) (Fuerst 1987; Owen 2000; Cummins et al. 2011;) due to greater GSH/hGSH content, GST activity with herbicide substrates, or both than sensitive species (Breaux 1987; Hatton et al. 1996). Our results with S-metolachlor are in accord with natural corn tolerance via GST activity (Fuerst 1987), but the elevated specific GST activities of CHR and SIR relative to WUS do not appear to be the primary resistance mechanism.

Plant P450s are integral membrane proteins in the ER that catalyze the insertion of one oxygen atom into lipophilic substrates (Werk-Reichhart and Feyereisen 2000; Siminszky 2006; Dimaano and Iwakami 2020). Microsomal P450s isolated from sorghum, corn and mung bean (Vigna radiata L. cv ‘Berken’) metabolize metolachlor via O-demethylation (Moreland et al. 1990; 1993; 1995). Our results clearly demonstrate that microsomes from CHR and SIR O-demethylate S-metolachlor and, importantly, to a much greater extent than microsomes from WUS or corn. P450s require an electron donor (typically NADPH) and ER-bound cytochrome P450 reductase activity (Werk-Reichhart and Feyereisen 2000; Siminszky 2006). The requirement of NAPDH for formation of O-demethylated S-metolachlor by CHR and SIR microsomes strongly supports P450-mediated O-demethylation activity. Previous metabolic assays with S-metolachlor using the P450 inhibitor malathion indirectly suggested P450 involvement in VLCFA inhibitor-resistant waterhemp (Strom et al. 2020). Our current results directly demonstrate that CHR and SIR microsomes oxidize S-metolachlor.

LC-MS experiments identified both O-demethylated S-metolachlor and GSH-S-metolachlor conjugates in waterhemp extracts, which support our results with in vitro enzyme assays. After conjugation, GSH-herbicide conjugates are typically transported into the vacuole by ABC transporters (Bartholomew et al. 2002; Riechers et al. 2010). GSH conjugates are catabolized inside the vacuole, yielding various dipeptide and CYS conjugates (Lamoureux and Rusness 1981; 1993; Wolf et al. 1996; Martin et al. 2007). The relative abundance of GSH, CYS-GLY, and CYS S-metolachlor conjugates was either equal, or more abundant, in WUS compared with CHR or SIR. By contrast, O-demethylated S-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates were more abundant in CHR and SIR than WUS.

The difference in abundances of S-metolachlor, O-demethylated S-metolachlor, and their metabolites among CHR, SIR, and WUS demonstrates the metabolic pathway of S-metolachlor.
in resistant waterhemp differs from sensitive waterhemp. Sensitive waterhemp primarily utilizes GSH conjugation for S-metolachlor detoxification, whereas multiple Phase I and II detoxification enzymes are involved in resistant waterhemp. Identification of O-demethylated S-metolachlor-GSH conjugates indicates that P450s and GSTs metabolize S-metolachlor independently or sequentially. O-demethylation was previously considered a secondary metabolic reaction for S-metolachlor in plants (Coleman et al. 2002), but our findings indicate greater importance in resistant waterhemp. Although it is not known if O-demethylated S-metolachlor inhibits VLCFA elongase activity, it is possible that rapid GST-catalyzed conjugation (in addition to other Phase II reactions; Fig. 7) of this key oxidative metabolite may contribute to complete detoxification of S-metolachlor in resistant waterhemp. Greater abundance of presumed glucose conjugates in resistant waterhemp supports previous findings of Phase II conjugation in plants following Phase I reactions (Moreland et al. 1990; Werk-Reichhart and Feyereisen 2000; Siminszky 2006), and implies that S-metolachlor detoxification in resistant waterhemp involves the concerted activities of multiple enzymes. By contrast, most plant detoxification pathways utilize either Phase I oxidation followed by Phase II sugar conjugation or Phase II GSH conjugation without a Phase I activation step (Kreuz et al. 1996). A unique exception is the complex metabolic pathway of chlorimuron-ethyl in corn, which exhibited both glucose and GSH conjugation of hydroxylated chlorimuron-ethyl (Lamoureux et al. 1991).

In many cases of weed resistance conferred by rapid herbicide metabolism, the same detoxification enzymes and pathways are utilized by tolerant crops in which the herbicide is applied (Anderson and Gronwald 1991; Ma et al. 2013; Tanetani et al. 2013; Yu and Powles 2014; Ducker et al. 2019). However, this is not always the case. For example, SIR formed ring-hydroxylated metabolites of the HPPD-inhibitor topramezone while corn generated a N-demethylated product (Lygin et al. 2018), although both reactions are likely catalyzed by P450(s). Our research demonstrates that the metabolic pathway for S-metolachlor in CHR and SIR is more intricate than corn or WUS, with multiple enzymes acting in concert to facilitate detoxification. Our results also indicate enhanced herbicide metabolism and non-target-site resistance are not always conferred by a single metabolic enzyme or enzyme family.
Conclusions

S-metolachlor has remained one of the most widely used herbicide active ingredients for weed management in corn and soybean since commercialization in the late 1990s (Blaser et al. 1999; USDA 2020). Metabolic resistance to S-metolachlor, however, reduces the duration and effectiveness of S-metolachlor for control of VLCFA inhibitor-resistant waterhemp populations (Hager 2019; Strom et al. 2019). Presently, the extent of VLCFA-inhibitor resistance in waterhemp and Palmer amaranth populations is unknown, but the biology and reproductive efficiency of these dioecious amaranths favor continued resistance evolution and spread of resistance alleles (Liu et al. 2012; Sarangi et al. 2017; Bagavathiannan and Davis 2018). Coupled with the rapid growth rate, C4 physiology, abiotic stress tolerance, and competitive ability of weedy dioecious amaranths (Ehleringer 1983; Steckel 2007; Chandi et al. 2013; Ward et al. 2013), multiple herbicide-resistant populations present a formidable pest management challenge. Therefore, integrating all viable chemical and non-chemical management practices is essential to control resistant populations, and will only be sustainable if new seed contributions to the soil-seedbank are limited each season (Buhler et al. 1997; Schwartz-Lazaro and Copes 2019; Geddes and Davis 2021).

Our present research findings present novel opportunities for the development of new herbicide chemistries and recommendations for growers. For example, understanding the intricacies of S-metolachlor metabolism in waterhemp provides insight into novel approaches for weed management, such as silencing resistance genes, inhibiting metabolic enzymes, or developing resistance-breaking chemistries (Kaundun 2021). Additionally, the enzyme assays developed for investigating S-metolachlor resistance in waterhemp will be useful for studying GSTs and P450s associated with herbicide resistance, environmental stress responses, adaptive mechanisms, and primary and secondary metabolism in *Amaranthus* and related species (Bhuiyan et al. 2007; Tanaka et al. 2008). Future metabolomic and lipidomic profiling of resistant *Amaranthus* populations will focus on identifying endogenous defense compounds and signaling metabolites that enable these pernicious weeds to successfully withstand stressful growing conditions.
Materials and Methods

Chemicals
Radiolabeled [URL-\(^{14}\)]C S-metolachlor (555 MBq mmol\(^{-1}\)) was supplied by Syngenta Crop Protection (Greensboro, North Carolina, USA). Non-labeled analytical grade S-metolachlor (98% pure; Fig. 1A) was purchased from Chem Service, Inc. (West Chester, Pennsylvania, USA). All other analytical grade chemicals and reagents were purchased through Fisher Scientific (Thermo-Fisher, Hanover Park, Illinois) or Sigma Chemical (Millipore Sigma, St. Louis, Missouri, USA).

Waterhemp populations and plant materials
Two previously-characterized multiple herbicide-resistant waterhemp populations (CHR and SIR) were selected. CHR originated from Champaign County, IL (Evans et al. 2019). SIR originated from McLean County, IL and is a subpopulation analogous to the MCR population described in previous research (Hausman et al. 2011, 2013; Ma et al. 2013). Both populations are resistant to VLCFA-inhibiting herbicides due to enhanced herbicide metabolism (Strom et al. 2020). The WUS population is sensitive to VLCFA-inhibiting herbicides (Strom et al. 2019) and was chosen for comparison. Corn (hybrid B73 x Mo17) was included for comparison due to natural corn tolerance to S-metolachlor via rapid GST-mediated metabolism (Fuerst 1987; Owen 2000). All waterhemp seeds were surface sterilized with bleach (50% v/v in water) for 10 min, rinsed twice in deionized water, and subsequently stratified in 0.1% agarose at 4°C for at least 20 d prior to initiating experiments to improve germination (Bell et al. 2013).

All plants were grown under greenhouse conditions (28/22°C day/night with a 16 h photoperiod) in 509 cm\(^3\) cell inserts containing vermiculite saturated with a commercial hydroponic fertilizer solution (Peters Hydroponic Special 5-11-26; ICL Specialty Fertilizers, Summerville, South Carolina, USA) at one-third strength supplemented with 0.15 g L\(^{-1}\) Ca(NO\(_3\))\(_2\). Natural sunlight was supplemented with mercury halide lamps to provide 800 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) photon flux at the soil surface. New hydroponic solution was added every two d until plants attained the desired growth stage. For all experiments, waterhemp seedlings were harvested...
when the second true leaf had fully expanded (approximately 2–3 cm), while corn shoots were harvested prior to the first leaf unfurling (approximately 4–5 cm). For enzymatic assays, whole waterhemp seedlings and corn shoots were then rinsed in deionized water to remove vermiculite, patted dry, sorted into 1 g fresh weight samples, placed in containers and frozen in liquid N\textsubscript{2}. Samples were stored at -80°C until experiments were initiated. Samples for all liquid chromatography-mass spectrometry (LC-MS) and β-glucosidase hydrolysis experiments consisted of ten waterhemp seedlings (approximately 0.25 g).

**Tissue homogenization and protein preparation for GST-activity assays**

Frozen 1 g samples were pulverized in a mortar pre-chilled with liquid N\textsubscript{2} and protein was extracted in 3 volumes of extraction buffer (200 mM Tris-Cl (pH 7.8), 1 mM Na\textsubscript{2}EDTA, 5 mM 2-mercaptoethanol, plant protease inhibitor cocktail (Protease Inhibitor Cocktail VI; bioWORLD, Dublin, Ohio, USA), and polyvinylpolypyrrolidone (PVPP; 75 mg mL\textsuperscript{-1})). Samples were then centrifuged at 15,000 \textit{x} g for 20 min at 4°C and the supernatant (crude protein extract) was removed and transferred to a 15 mL centrifuge tube. Crude protein extracts were then precipitated by adjusting the final volume to 80% (v/v) acetone at 4°C (Lester \textit{et al}., 2004). Acetone precipitation was conducted to concentrate protein from each tissue sample. Precipitated protein was then re-centrifuged at 15,000 \textit{x} g for an additional 10 min at 4°C and the supernatant was decanted. Acetone-precipitated protein was gently dried on ice with N\textsubscript{2} gas and stored at -80°C.

On the same day that protein samples were used in enzymatic assays, precipitated protein was resuspended in the same extraction buffer described previously (0.5 mL waterhemp; 0.8 mL corn) without PVPP. Protein was resuspended by gently stirring with a paintbrush dipped in buffer and remaining plant pigments were removed with a desalting column (Zeba Spin Desalting Column, MWCO 7000 Da, ThermoFisher Scientific, Waltham, Massachusetts, USA) preconditioned in three column volumes of protein extraction buffer without PVPP. Protein concentrations within samples was determined by a Bradford assay (Bradford, 1976) utilizing a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).
**Waterhemp and corn GST-activity assays with S-metolachlor**

GST activity assays with S-metolachlor were modified from previous studies with the herbicides atrazine (Evans et al. 2017) or dimethenamid (Riechers et al. 1996b). Reaction mixtures consisted of 100 mM Tris-Cl (pH 7.8), 10 mM GSH, partially purified protein extracts (0.1 mg assay\(^{-1}\)), and 0.2 mg mL\(^{-1}\) BSA. Negative controls were included to determine the non-enzymatic conjugation rate of GSH with S-metolachlor and protein was replaced by extraction buffer without PVPP. Assay mixtures were incubated for five min at 30°C for equilibration then reactions were initiated by adding radiolabeled S-metolachlor (100 µM final concentration; 29.6 MBq mmol\(^{-1}\) specific radioactivity) to adjust the final volume to 500 µL. Reactions were incubated for 30 min at 30°C and were terminated by the addition of 50 µL glacial acetic acid.

Reactions mixtures were partitioned once against 900 µL methylene chloride to separate unconjugated S-metolachlor from its polar GSH-S-metolachlor metabolite. Samples were vortexed and briefly centrifuged at 10,000 \(x\) g. The following day, radioactivity was determined in 200 µL of the aqueous phase by liquid scintillation spectrometry (LSS). Approximately 4 and 6% of radiolabeled S-metolachlor was recovered as the GSH-S-metolachlor metabolite in waterhemp and corn GST assays, respectively. Enzymatic conjugation rates were determined by subtracting the average radioactivity from the aqueous phase of negative control reactions (no protein added) from the total activity measured in experimental reactions. Specific activity was determined based on protein concentration and the corrected total activity determined by LSS. Units of GST activity are reported as pmol GSH-S-metolachlor conjugate mg protein\(^{-1}\) min\(^{-1}\). GST activity results represent the combined data from three independent experiments with two protein extracts per population per experiment and three technical replications per protein extract.

**Waterhemp and corn microsome preparation**

Frozen tissue samples (3 g) were pulverized in a mortar pre-chilled with liquid N\(_2\) and protein was extracted in 5 volumes of extraction buffer (200 mM Tris-Cl (pH 7.8), 1 mM \(\text{Na}_2\text{EDTA}\), 250 mM sucrose, 40 mM ascorbic acid, 5 mM dithiothreitol (DTT), plant protease inhibitor cocktail (described above), and PVPP (75 mg mL\(^{-1}\))). Samples were then centrifuged at 15,000 \(x\) g for 20
min at 4°C and the supernatant (crude protein extract) was removed, filtered through cheese cloth, and transferred to a 10.4 mL ultracentrifuge tube (16 x 76 mm polycarbonate centrifuge bottle; Beckman Coulter Life Sciences, Brea, California, USA). Samples were then centrifuged at 100,000 x g for 60 min at 4°C (Optima LE-80K Ultracentrifuge, Beckman Coulter Life Sciences). The soluble portion (supernatant) was discarded and the high-speed pellet containing the microsomal protein was rinsed three times with 1 mL of 200 mM Tris-Cl (pH 7.8) and resuspended in 330 µL of ice-cold storage buffer (200 mM Tris-Cl (pH 7.8), 3 mM DTT, and 30% (v/v) glycerol) by gently stirring the pellet with a paintbrush previously dipped in storage buffer. Microsomes were then stored on ice until utilized for enzyme assays the same day. Protein concentrations were determined (Bradford 1976) utilizing a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

**Oxidation of S-metolachlor by waterhemp and corn shoot microsomes**

Microsomal activity assays with radiolabeled S-metolachlor were conducted *in vitro* similarly to previous studies with several herbicide substrates and crops (Moreland et al. 1990; 1993; 1995; Polge and Barrett 1995) but with modifications for waterhemp. Preliminary experiments (Supplementary Fig. S2) were performed to determine the most favorable reaction pH, reaction time, and electron donor as well as optimize product formation for detection via high performance liquid chromatography (HPLC). Specific P450 activities of microsomes extracted from CHR and assayed at pH values of 7.4 and 7.8 were equal but greater than at pH 7.1 (Supplementary Fig. S2A), so pH 7.4 was utilized in subsequent experiments. Reaction times tested at 30°C between 30–90 min demonstrated that specific activity was highest at 30 min, then steadily declined (Supplementary Fig. S2B). Although total activity increased after 30 min, specific activity decreased after 30 min, so a reaction time of 25 min was ultimately chosen to balance relatively high specific activity (Supplementary Fig. S2C) with detectable product formation via HPLC. Several concentrations of NADPH (Cayman Chemicals, Ann Arbor, Michigan, USA) and NADH (Research Products Intl., Mount Prospect, Illinois, USA) were tested as electron donors (alone or in combination), but a 1 mM NADPH concentration was optimal (Supplementary Fig. S2D).
Following preliminary assay optimization, final reaction mixtures consisted of 100 mM Tris-Cl (pH 7.4), 5 µM radiolabeled S-metolachlor (555 MBq mmol⁻¹), and microsomal protein (0.1 mg waterhemp; 0.2 mg corn). Assays were incubated for five min at 30°C to equilibrate then reactions were initiated by addition of NADPH (1 mM final concentration), adjusting the final assay volume to 500 µL. Reactions were incubated for 25 min at 30°C, terminated by the addition of 50 µL glacial acetic acid, then frozen in liquid N₂ and stored at -20°C. Reaction mixtures were partitioned three times with 1 mL ethyl acetate. Samples were vortexed and briefly centrifuged at 10,000 x g to denature protein at the interface of the aqueous and organic phases. The three separate organic phases were transferred to a 5 mL Eppendorf tube, and following the third partitioning, ethyl acetate was evaporated under N₂ gas. Each sample was then reconstituted in 100 µL acetonitrile (50% v/v) prior to conducting HPLC. Percent recovery of radiolabeled compounds exceeded 95% and radioactivity was not detected in the aqueous phase following partitioning. HPLC analysis (described below) confirmed that a single major reaction product, radiolabeled O-demethylated S-metolachlor, was present in the organic phase.

**Quantification of microsome-generated S-metolachlor metabolite**

Reverse-phase HPLC was performed with a PerkinElmer Flexar LC system (Model N2910401, Perkin Elmer, Akron, Ohio) at a flow rate of 1 mL min⁻¹ using a C18 column (Nucleodur C18 Pyramid, 5 µm x 250 mm; Macherey-Nagel, Düren, Germany). Eluent A was water with 0.1% (v/v) formic acid and eluent B was acetonitrile with 0.1% (v/v) formic acid. Sample volumes of 77 µL were injected and extractable radioactivity was resolved with a 19 min stepwise gradient starting at 90:10 A:B. Steps included a linear gradient to 75:25 A:B in 5 min, 60:40 A:B in 3 min, 40:60 A:B in 2 min, 15:85 A:B in 2 min, and finally 5:95 A:B in 3 min followed by a 4 min isocratic hold. Following each 19 min run, the column was returned to 90:10 A:B in 5 min and equilibrated for 3 min before subsequent injections.

Radiolabeled compounds were detected with a β-RAM Radio HPLC-detector (Model 4; LabLogic Systems, Tampa, Florida, USA) and Ultima-Flow M cocktail (PerkinElmer). The amount of O-demethylated S-metolachlor in each assay was determined by peak integration in Laura.
Software (Version 4.2.3.37; LabLogic Systems). Peaks were then compared by co-chromatography to non-labeled O-demethylated S-metolachlor (supplied by Syngenta Ltd., UK) (Fig. 1B). Specific activity was determined based on protein concentration and the amount of radiolabeled O-demethylated S-metolachlor formed per assay. Units of microsomal activity are reported as pmol O-demethylated S-metolachlor mg protein$^{-1}$ min$^{-1}$. A third assay for each microsomal protein extract was included with water substituted for NADPH to determine if product formation was NADPH dependent. In addition, non-enzymatic reactions were included in each experiment by replacing microsomal protein with storage buffer to determine if non-enzymatic oxidation of S-metolachlor had occurred; however, non-enzymatic oxidation was not detected. Peak retention times ($R_T$) for S-metolachlor and O-demethylated S-metolachlor were 17.4 and 16.0 min, respectively. Microsomal activity results represent the combined data from three independent experiments with two protein extracts per population per experiment and two assays per protein extract.

**Untargeted liquid chromatography-mass spectrometry**

Greenhouse-grown plants were transferred to a growth chamber set at 28/22°C day/night (16 h photoperiod) 24 h prior to experiments to allow plants to equilibrate to growth chamber conditions. Fluorescent and incandescent lights provided 550 µmol m$^{-2}$ s$^{-1}$ photon flux at the plant canopy level. Samples of ten waterhemp seedlings or five corn shoots were washed with deionized water and placed in a 5 mL Eppendorf tube containing 1 mL of hydroponic solution plus 100 µM non-labeled S-metolachlor for 2 h. At 2 h, plant samples were triple rinsed with deionized water, fresh weights were recorded, frozen in liquid nitrogen, and stored at -80°C until processing. Plant samples for additional time points (> 2 h) were triple rinsed with deionized water, then transferred to a 60 mL plastic cup per population containing 2 mL of fresh hydroponic solution without herbicide. Plants were then removed, frozen, and stored at 4 and 12 h after treatment. Additional samples were treated using the same methods, but S-metolachlor was excluded during the 2 h treatment process to enable comparisons of the metabolome from treated and non-herbicide treated samples.
Following incubation, frozen tissue samples (approx. 0.25 g) were ground in liquid N\textsubscript{2} and compounds were extracted in 1 mL of 90\% (v/v) methanol. After the first extraction and centrifugation at 12,000 \(x\) g, the supernatant was removed and remaining plant material was re-extracted in an additional 1 mL of 90\% (v/v) methanol. Following centrifugation, the pellet was discarded, supernatant removed and combined with the first supernatant, yielding a total final volume of 2 mL. Quality control samples were then prepared by combining aliquots of each sample (treated and nontreated) for injection throughout each experimental run (Dunn et al. 2011). Synthetic standards of \(S\)-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates along with \(O\)-demethylated \(S\)-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates were included (refer to Fig. 7 for structures). Standards were synthesized \textit{in vitro} (described below) or supplied by Syngenta. All samples were stored at -80°C until further analysis.

Samples were submitted to the Roy J. Carver Metabolomics Facility at the University of Illinois for analysis using methods previously described (Elolimy et al. 2019). Prior to analysis, all samples were spiked with 4-chloro-DL-PHE as an internal standard. Samples were then analyzed with a Q-Exactive MS system (Thermo, Bremen, Germany). LC separation was conducted with a Dionex Ultimate 3000 series HPLC equipped with a Phenomenex C18 column (4.6 x 100mm, 2.6 \(\mu\)m). Mobile phases consisted of A (\(H_2O\) with 0.1\% formic acid (v/v)) and B (acetonitrile with 0.1\% formic acid (v/v)). The flow rate was set at 0.25 mL min\(^{-1}\) with linear gradient starting at 100 \% A for 3 min. The gradient then transitioned to 100\% B (20–30 min) and returned to 100\% A (31–36 min). Twenty \(\mu\)L of each sample was injected and the autosampler temperature was set at 15°C. Mass spectra were then acquired under both positive (sheath gas flow rate: 45; aux gas flow rate: 11; sweep gas flow rate: 2; spray voltage: 3.5 kV; capillary temp: 250°C; Aux gas heater temp: 415°C) and negative electrospray ionization (sheath gas flow rate: 45; aux gas flow rate: 11; sweep gas flow rate: 2; spray voltage: −2.5 kV; capillary temp: 250°C; Aux gas heater temp: 415°C). The full scan mass spectrum resolution was set to 70,000 with scan range of \(m/z\) 67 ~ \(m/z\) 1,000, and AGC target was 1E6 with a maximum injection time of 200 ms.
LC-MS data were further analyzed with Thermo Compound Discoverer software (v. 2.1 SP1) for chromatographic alignment and compound feature identification and quantitation. Multivariate analysis of peak areas of metabolite features detected in at least 60% of the QC samples was then conducted using unsupervised principal component analysis (PCA) in SIMCA software v.15 (Umetrics, Sweden) to visualize trends in the metabolomics data between resistant and sensitive waterhemp populations. Prior to analysis, values were log transformed and Pareto scaled to minimize the disequilibrium between metabolites produced at high and low concentrations within the waterhemp seedlings (van den Berg et al. 2006).

**Targeted, high-resolution liquid chromatography-mass spectrometry**

Plants were initially treated and processed as described above for untargeted LC-MS experiments. During the first extraction of compounds in 90% (v/v) methanol, 100 µL of 100 µM acetochlor (a similar chloroacetamide) was added to each sample as an internal standard (Supplementary Fig. S1). Samples were then analyzed at the Roy J. Carver Metabolomics Facility at the University of Illinois. Standards of ten S-metolachlor metabolites (structures shown in Supplementary Fig. S1 and Fig. 7) were either supplied by Syngenta or synthesized *in vitro* (described below). Metabolites were the same as investigated in untargeted experiments, with the addition of the γ-GLU-CYS conjugates of S-metolachlor and O-demethylated S-metolachlor.

LC-MS separation was conducted using the same method described for untargeted experiments (Elolimy et al. 2019), except that the 4-chloro-DL-PHE was not included. Peak areas were then determined for each of the included standards in Xcalibur Software 4.1.31.9 (Thermo, Bremen, Germany). Relative values for each standard from treated plant samples were calculated by dividing the peak area of each metabolite by the peak area of acetochlor in each sample. The quotient was then adjusted for the fresh weight of each sample, yielding a relative abundance of each metabolite among populations, similar to previous experiments with the herbicide topramezone (Lygin et al. 2018). Comparisons of peak areas of S-metolachlor and its metabolites among waterhemp populations and HAT were carried out on LC-MS data using the lme package in R (version 4.0.0) and RStudio (Version 1.2.1335).
Assumptions of normality of residuals were tested via the Shapiro-Wilk test and inspection of quantile-quantile plots. Normalization was carried out using Tukey's ladder of powers method in rcompanion package (version 2.4.1). The mixed models included population, HAT, and their interaction as fixed effects and experiment and replicates nested within experiment as random effects. Analysis of variance was conducted with an $\alpha = 0.05$. Targeted LC-MS experiments were conducted twice with three replications per population per time-point.

**Qualitative analysis of putative glucose conjugates**

Plants were grown and prepared as previously described (Strom et al. 2020), but the incubation step was modified to include 100 µM radiolabeled S-metolachlor (11.2 kBq mL$^{-1}$; 111 MBq mmol$^{-1}$ specific activity). Plants were incubated for 2 h in S-metolachlor-treated hydroponic solution. At 2 h, samples were triple rinsed in deionized water and transferred to a new 60 mL plastic cup containing 2 mL of nontreated hydroponic solution. At 12 h after treatment, plant samples were triple rinsed in deionized water, fresh weights recorded, frozen in liquid N$_2$, and stored at -20°C until further analysis.

Radioactivity was extracted twice in 90% (v/v) methanol as described previously (Strom et al. 2020). The methanolic extract was then evaporated to incipient dryness under N$_2$ gas and reconstituted in 500 µL of 50% acetonitrile (v/v). Radioactivity was then quantified by LSS. Enzyme assays were then conducted to determine if glucose conjugates of S-metolachlor metabolites were present in extracts from the CHR, SIR, and WUS populations by subjecting them to $\beta$-glucosidase hydrolysis, using methods modified from Edwards (1994). Assays included 6 mg mL$^{-1}$ $\beta$-glucosidase (from sweet almond, >4 U mg$^{-1}$; Sigma-Aldrich), 0.2 µg mL$^{-1}$ BSA, concentrated plant extract containing radiolabeled S-metolachlor and its metabolites (15,000 dpm; 250 Bq) in 50 mM sodium phosphate buffer (pH 6.0) and a final volume of 2.0 mL. Assays were incubated at 37°C for 24 h, then terminated by freezing in liquid N$_2$. Control reactions were also performed under the same conditions but without $\beta$-glucosidase to compare metabolite profiles between hydrolyzed and non-hydrolyzed samples via HPLC. Experiments were conducted twice, with three replications per population per treatment.
Prior to reverse-phase HPLC analysis, samples were purified and concentrated by solid-phase extraction (SPE) using a preparative (3 mL) C18 column (200 mg; Chromabond C18ec; Machery-Nagel, Düren, Germany). SPE columns were pre-conditioned with 2 volumes of methanol followed by 2 volumes of water acidified with 0.1% formic acid. Samples were then loaded onto the column under vacuum at 1 mL min\(^{-1}\), washed with 1 mL of acidified water, dried under vacuum, and metabolites eluted in 2 x 0.5 mL methanol. Methanol was then evaporated under N\(_2\) gas and samples were reconstituted in 100 µL of 50% (v/v) acetonitrile.

Reverse-phase HPLC was performed similar to microsomal assays, but radiolabeled compounds were resolved with a longer stepwise gradient (26 min) starting at 90:10 A:B to improve the resolution of polar metabolites. Steps included a linear gradient to 75:25 A:B in 5 min, 60:40 A:B in 5 min, 40:60 A:B in 5 min, 15:85 A:B in 5 min, and finally 5:95 A:B in 3 min followed by a 3 min isocratic hold. Following each 26 min run, the column was returned to 90:10 A:B in 4 min and equilibrated for 3 min before subsequent injections. Radiolabeled compounds were detected with a β-RAM Radio HPLC-detector (Model 4; LabLogic Systems) and Ultima-Flow M cocktail (PerkinElmer). Chromatograms of each sample were then analyzed and S-metolachlor metabolites were compared between β-glucosidase-treated and nontreated reactions in Laura Software (Version 4.2.3.37; LabLogic Systems).

**In vitro formation of synthetic standards**

Synthetic GSH, CYS-GLY, γ-GLU-CYS and CYS conjugates of S-metolachlor and O-demethylated S-metolachlor were prepared in vitro as previously described with the herbicide dimethenamid (Riechers et al. 1996a). Reactions were carried out in 2 mL Eppendorf tubes containing 60 mM TAPS ([Tris(hydroxymethyl)methylamino]propanesulfonic acid) buffer (unadjusted pH 8.5), 10 mM GSH, dipeptide, or L-CYS, and 20 µM S-metolachlor or O-demethylated S-metolachlor in 1 mL of deionized water. Reaction mixtures were incubated for 24 h at 35°C and then terminated by adjusting the final volume to 1.5% formic acid. Following termination, reaction mixtures were partitioned once against 1 volume of methylene chloride to remove non-conjugated S-metolachlor or O-demethylated S-metolachlor. The conjugates of S-metolachlor or O-demethylated S-metolachlor in the aqueous phase were purified and concentrated by SPE using...
a preparative (3 mL) C18 column (200 mg; Chromabond C18ec; Machery-Nagel, Düren, Germany). Columns were pre-conditioned with 2 volumes of methanol followed by 2 volumes of acidified water (0.1% formic acid). The aqueous phase was then loaded onto the column under vacuum at 1 mL min\(^{-1}\), washed with 1 mL of acidified water, dried under vacuum, and synthetic conjugates were eluted in 2 x 0.5 mL methanol. The methanol was then evaporated under a stream of nitrogen gas and standards were reconstituted in 200 μL of 90% methanol for use as analytical standards in LC-MS.

**Statistical analysis**

Specific activities from GST and microsomal assays with S-metolachlor as substrate were analyzed in SAS 9.4 (SAS Institute, Inc., Cary, North Carolina, USA). The assumptions of homogenous variance and normality of residuals were reviewed with PROC UNIVARIATE and PROC GLM, respectively. Data from GST and microsomal assays were then analyzed by a generalized linear mixed model in PROC GLIMMIX fitted with a normal distribution and an identity link function. The response variable was pmol (GSH-S-metolachlor conjugate or O-demethylated S-metolachlor) mg protein\(^{-1}\) min\(^{-1}\) with population as a fixed effect. Random effects included experiment run, protein sample nested within run, and assay nested within protein sample and experimental run. Mean separations were then conducted by least significant difference (\(\alpha = 0.05\)).

**Supplementary data**

Supplementary data are available at PCP online.

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**Data availability**

Data are available upon request.
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Disclosure
The authors have no conflict of interest to declare.

Author contributions
DER, AGH, SSK, and SAS conceptualized the work. SAS and DER designed the experiments, and SAS implemented the experiments, summarized and interpreted results, and prepared the manuscript. SSK and JAM provided seed, analytical standards, and technical assistance regarding S-metolachlor degradation. JCTC assisted with LC-MS experiment initiation and analysis. ASD, NJS, and JCTC assisted with statistical analysis, and all listed authors conducted critical reviews of the final manuscript draft.
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### Tables

**Table 1.** Glutathione S-transferase activities from partially purified extracts of three waterhemp (*Amaranthus tuberculatus*) populations and corn (*Zea mays*), measured using radiolabeled S-metolachlor as substrate.

| Population | Protein Per Assay | Total Activity<sup>a</sup> | Specific Activity<sup>b</sup> |
|------------|------------------|-----------------------------|-------------------------------|
| CHR        | 0.1 mg           | 434 pmol                    | 138 pmol mg<sup>-1</sup>min<sup>-1</sup> |
| SIR        | 0.1 mg           | 462 pmol                    | 156 pmol mg<sup>-1</sup>min<sup>-1</sup> |
| WUS        | 0.1 mg           | 265 pmol                    | 79 pmol mg<sup>-1</sup>min<sup>-1</sup> |
| Corn<sup>c</sup> | 0.1 mg       | 1320 pmol                   | 403 pmol mg<sup>-1</sup>min<sup>-1</sup> |

<sup>a</sup>Total activity represents pmol GSH-S-metolachlor conjugate formed during a 30 min assay.

<sup>b</sup>Mean specific activities were separated by Fisher’s LSD and values followed by the same letter are not significantly different (α=0.05).

<sup>c</sup>Corn (hybrid B73 x Mo17) was included to represent a tolerant crop.

**Table 2.** Activity of microsomal extracts from three waterhemp (*Amaranthus tuberculatus*) populations and corn (*Zea mays*), measured using radiolabeled S-metolachlor as substrate.

| Population | Protein Per Assay | Total Activity<sup>a</sup> | Specific Activity<sup>b</sup> |
|------------|------------------|-----------------------------|-------------------------------|
| CHR        | 0.1 mg           | 105 pmol                    | 51 pmol mg<sup>-1</sup>min<sup>-1</sup> |
| SIR        | 0.1 mg           | 137 pmol                    | 66 pmol mg<sup>-1</sup>min<sup>-1</sup> |
| WUS        | 0.1 mg           | 5.5 pmol                    | 2.4 pmol mg<sup>-1</sup>min<sup>-1</sup> |
| Corn<sup>c</sup> | 0.2 mg       | 6.7 pmol                    | 1.7 pmol mg<sup>-1</sup>min<sup>-1</sup> |

<sup>a</sup>Total activity represents pmol O-demethylated S-metolachlor formed during a 25 min assay.

<sup>b</sup>Mean specific activities were separated by Fisher’s LSD and values followed by the same letter are not significantly different (α=0.05).

<sup>c</sup>Corn (hybrid B73 x Mo17) was included to represent a tolerant crop.
Figure legends

Figure 1. Structures of parent S-metolachlor (A) and O-demethylated S-metolachlor (B).

Figure 2. Representative reverse-phase HPLC chromatograms from microsomal assays of VLCFA-inhibitor-resistant (A, CHR; B, SIR) and sensitive (C, WUS) waterhemp (Amaranthus tuberculatus) seedlings or corn (Zea mays) shoots (D) after 25 min incubation. Regions shaded in red in each panel correspond to O-demethylated S-metolachlor, peak retention time ($R_T$) of 16 min. Green-shaded regions correspond to S-metolachlor ($R_T = 17.4$ min). Panel E, non-enzymatic reaction. Panel F, VLCFA-inhibitor-resistant (SIR) waterhemp without NADPH.

Figure 3. Principal components analysis of the metabolite features detected in VLCFA-inhibitor-resistant (CHR, SIR) and sensitive (WUS) waterhemp (Amaranthus tuberculatus) seedling extracts. Panel A, Scores plots corresponding to waterhemp populations colored according to populations. Scores are labeled according to h after treatment in which the sample was harvested. Panel B, Loadings plot of all metabolites ($X$ variables represented by green circles) detected in VLCFA-inhibitor-resistant (CHR, SIR) and sensitive (WUS) waterhemp (Amaranthus tuberculatus) seedling extracts. Metabolites highlighted in red correspond to compounds identical to S-metolachlor metabolite standards.

Figure 4. Relative abundance of S-metolachlor and its respective GSH, $\gamma$-GLU-CYS, CYS-GLY, and CYS conjugates from extracts of VLCFA-inhibitor-resistant (CHR and SIR) and sensitive (WUS) waterhemp (Amaranthus tuberculatus) populations. Relative values were determined by dividing the peak area of each metabolite by the peak area of the internal standard (acetochlor) and the fresh weight of each sample. Significant differences based on $p$-values were obtained from ANOVA by comparing the relationships between fixed effects [population, hour after treatment (HAT), and their interaction (Pop x HAT)] for each metabolite, and are indicated as follows: *** = $p<0.001$; ** = $p<0.01$; * = $p<0.05$; ns = not significant.
Figure 5. Relative abundance of O-demethylated S-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates from extracts of VLCFA-inhibitor-resistant (CHR and SIR) and sensitive (WUS) waterhemp (*Amaranthus tuberculatus*) populations. Relative values were determined by dividing the peak area of each metabolite by the peak area of the internal standard (acetochlor) and the fresh weight of each sample. Significant differences based on p-values were obtained from ANOVA by comparing the relationships between fixed effects [population, hour after treatment (HAT), and their interaction (Pop x HAT)] for each metabolite, and are indicated as follows: *** = p<0.001; ** = p<0.01; * = p<0.05; ns = not significant.

Figure 6. Effect of *in vitro* β-glucosidase hydrolysis on representative metabolite profiles of waterhemp (*Amaranthus tuberculatus*) plant extracts, 12 h after treatment with radiolabeled S-metolachlor. Panel A, VLCFA-inhibitor-resistant (CHR) non-hydrolyzed extract; and Panel B, with β-glucosidase treatment. Panel C, VLCFA-inhibitor-resistant (SIR) non-hydrolyzed extract; and Panel D, with β-glucosidase treatment. Panel E, VLCFA-inhibitor-sensitive (WUS) non-hydrolyzed extract; and Panel F, with β-glucosidase treatment. Hydrolyzed plant extracts contained β-glucosidase at 6 mg mL⁻¹ (>4 U mg⁻¹) using a protocol modified from Edwards (1994) as described in Methods; non-hydrolyzed plant extracts were treated in the same manner but without the enzyme. Reverse-phase HPLC was used to resolve polar metabolites of radiolabeled S-metolachlor (gradient described in Methods). Blue-shaded regions correspond to a putative glucose conjugate (R<sub>T</sub> 3.3 min). Pink-shaded regions correspond to O-demethylated S-metolachlor (R<sub>T</sub> 19.4 min) and red-shaded regions correspond to S-metolachlor (R<sub>T</sub> 23.2 min).

Figure 7. Proposed metabolic detoxification routes of the very-long-chain fatty acid (VLCFA)-inhibiting herbicide, S-metolachlor, in VLCFA-inhibitor-resistant waterhemp (*Amaranthus tuberculatus*). Dotted lines indicate possible, but less likely or not yet investigated, enzymatic steps of S-metolachlor metabolism compared to reactions indicated by solid arrows.
Figure 3

A. PCA plot showing differentiation between S-metolachlor treated and non-treated groups.

B. PCA plot highlighting specific metabolites: S-metolachlor, S-metolachlor GSH, S-metolachlor CYS, O-demethylated S-metolachlor CYS, O-demethylated S-metolachlor CYS-GLY, O-demethylated S-metolachlor GSH.
Figure 4
Figure 5
