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Proteomic and biochemical assays of glutathione-related proteins in susceptible and multiple herbicide resistant *Avena fatua* L.

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ABSTRACT

Extensive herbicide usage has led to the evolution of resistant weed populations that cause substantial crop yield losses and increase production costs. The multiple herbicide resistant (MHR) *Avena fatua* L. populations utilized in this study are resistant to members of all selective herbicide families, across five modes of action, available for *A. fatua* control in U.S. small grain production, and thus pose significant agronomic and economic threats. Resistance to ALS and ACCase inhibitors is not conferred by target site mutations, indicating that non-target site resistance mechanisms are involved. To investigate the potential involvement of glutathione-related enzymes in the MHR phenotype, we used a combination of proteomic, biochemical, and immunological approaches to compare their constitutive activities in herbicide susceptible (HS1 and HS2) and MHR (MHR3 and MHR4) *A. fatua* plants. Proteomic analysis identified three tau and one phi glutathione *S*-transferases (GSTs) present at higher levels in MHR compared to HS plants, while immunoassays revealed elevated levels of lambda, phi, and tau GSTs. GST specific activity towards 1-chloro-2,4-dinitrobenzene was 1.2-fold higher in MHR4 than in HS1 plants and 1.3- and 1.2-fold higher in MHR3 than in HS1 and HS2 plants, respectively. However, GST specific activities towards fenoxaprop-P-ethyl and imazamethabenz-methyl were not different between untreated MHR and HS plants. Dehydroascorbate reductase specific activity was 1.4-fold higher in MHR than HS plants. Pretreatment with the GST inhibitor NBD-Cl did not affect MHR sensitivity to fenoxaprop-P-ethyl application, while the herbicide safener and GST inducer mefenpyr reduced the efficacy of low doses of fenoxaprop-P-ethyl on MHR4 but not MHR3 plants. Mefenpyr treatment also partially reduced the efficacy of thiencarbazone-methyl or mesosulfuron-methyl on MHR3 or MHR4 plants, respectively. Overall, the GSTs described here are not directly involved in enhanced rates of fenoxaprop-P-ethyl or imazamethabenz-methyl metabolism in MHR *A. fatua*. Instead, we propose that the constitutively elevated GST proteins and related enzymes in MHR plants are representative of a larger, more global suite of abiotic stress-related changes.

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1. Introduction

Herbicide resistance is a rapidly growing worldwide problem that causes significant crop yield losses, increases production costs, and threatens our ability to successfully manage weed populations [1,2]. Resistance can be conferred by target site overexpression or mutations that alter herbicide binding, or non-target site resistance (NTSR) mechanisms like enhanced rates of herbicide metabolism, reduced absorption/translocation, sequestration, or changes in generalized abiotic stress defense networks [3]. Multiple herbicide resistance (MHR), in which weeds are resistant to two or more unrelated herbicides, is a particularly ominous development in many cropping systems worldwide, and threatens the very basis of herbicidal weed management.

Avena fatua (Poaceae, Pooideae, Aveneae; wild oat) is an annual, allohexaploid, predominantly self-pollinating monocot that is ranked as one of the world's worst weed species [4]. It is particularly well-adapted to temperate regions of Europe and North America, and infests over 11 million ha of cropland in the Northern Great Plains alone [5]. *A. fatua* populations that are resistant to one or more herbicides have been reported in the USA [6–8] and elsewhere [1]. We recently described the *A. fatua* MHR3 and MHR4 populations that are resistant to the acetyl-CoA carboxylase (ACCase) inhibitors fenoxaprop-P-ethyl, tralkoxydim, and pinoxaden, the acetolactate synthase (ALS) inhibitors imazamethabenz-methyl and flucarbazone, the growth inhibitor difenzoquat, the photosystem I inhibitor paraquat (MHR3 only), and the very long chain fatty acid (VLCFA) biosynthesis inhibitor triallate, with resistant/susceptible ED_{50} ratios ranging from 1.4 to 57 [6,9]. The MHR *A. fatua* populations are thus resistant to members of all selective herbicide families available for *A. fatua* control in U.S. small grain production. Resistance to ALS- and ACCase-inhibiting herbicides is not

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due to known target site mutations, and the cytochrome P450 monooxygenase inhibitor malathion partially reversed the resistance phenotype for several herbicides [6], indicating that NTSR mechanisms are involved.

The central component of plant response to abiotic stresses like herbicides is activation of the xenome, or the xenobiotic detection, transport, and detoxification network [10]. Glutathione (GSH)-related enzymes such as glutathione *S*-transferases (GSTs) and others are critical xenome players that are involved in enhanced degradation rates of several herbicides (reviewed in [11,12]). Compounds like the herbicide safener and GST inducer mefenpyr [13,14] and the GST inhibitor 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) [15,16] have proven invaluable in dissecting the contributions of GSTs in herbicide metabolism and MHR.

Our previous work showing that enhanced metabolism was likely involved in *A. fatua* resistance to several herbicides [6] led us to investigate the potential involvement of GSH-related enzymes in the MHR phenotype. We used a combination of proteomic, biochemical, and immunological approaches to compare the enzymes of GSH conjugation and related activities in HS and MHR *A. fatua* plants.

2. Materials and methods

2.1. Plant material

The MHR3 and MHR4 populations were derived from seeds collected in 2006 from two *A. fatua* populations not controlled by 60 g a.i. ha^{-1} pinoxaden (Axial, Syngenta Crop Protection; ACCase inhibitor) in two production fields separated by approximately 8 km in Teton County, Montana, USA. Field-collected seeds (about 90% of which were resistant to 60 g a.i. ha^{-1} pinoxaden, data not shown) were subjected to two generations of recurrent group selection (50 plants each generation) by spraying with the same dose of pinoxaden, after which 100% of plants were confirmed to be homozygous resistant to pinoxaden via dose response experiments [6,17]. From each generation of 50 plants, all seeds were harvested and a random selection of 50 seeds was used to initiate the next generation. The herbicide susceptible population HS1 was derived from seeds collected from untreated border plants in an adjacent field, and was subsequently confirmed to be 100% susceptible to the herbicides used in these studies [17]. A second susceptible population, HS2, is the inbred nondormant SH430 line used in seed dormancy research [18,19]. Plants were grown under a 16-hr photoperiod of natural sunlight supplemented with mercury vapor lamps (165 μmol m^{-2} s^{-1}) at 25 ± 4 °C in standard greenhouse soil mix [1:1:1 (by vol) Bozeman silt loam:Sunshine mix #12 (Sun Gro Horticulture, Inc., Bellvue, WA):perlite] and fertilized weekly with Jack's water soluble 20 N-20 P-20 K (JR Peters Inc., Allentown, PA). All plants for each experiment were grown on the same greenhouse bench and were harvested in mid-morning to minimize environmental- and circadian-induced changes in protein levels.

2.2. GSH affinity chromatography

Shoot tissue (250 g) from fully tillered (BBCH stage 29) HS1 and MHR4 plants was ground under liquid nitrogen and suspended in ice cold extraction buffer (EB) containing 0.1 M Tris HCl (pH 7.5), 2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 5% (w/v) PVPP. The slurries were homogenized (Polytron Homogenizer, Brinkman Instruments, Westbury NY) for 4 min on ice and filtered through Miracloth (EMD Millipore, Merck KGaA, Darmstadt, Germany). The filtrates were centrifuged at 19,600 ×*g* for 20 min at 4 °C and the supernatants subjected to 0–80% ammonium sulfate precipitation at 0 °C. After centrifugation at 14,300 ×*g* for 15 min at 4 °C, the resulting protein pellets were redissolved in EB and dialyzed against the same buffer for 16 h at 4 °C. Following addition of Triton X-100 to 1% (v/v), protein samples (10 mg ml^{-1}) were subjected to GSH-agarose (Sigma-Aldrich G4510) affinity chromatography according to manufacturer's instructions. GSH-binding proteins were eluted with 10 mM reduced GSH, precipitated twice with acetone at −20 °C overnight and resuspended in DIGE buffer (7 M urea, 2 M thiourea, 30 mM Tris pH 8.5, 4% [w/v] CHAPS, 1X protease inhibitor and nuclease mix [GE Healthcare Life Sciences, Pittsburgh, PA], and 0.1% [w/v] bromophenol blue) at 2 mg ml^{-1}. Protein concentrations were determined [20] using bovine serum albumin (BSA) fraction V as standard.

2.3. 2D-DIGE

Two-dimensional difference gel electrophoresis (2D-DIGE) was initiated by minimal fluorescent labeling of lysine side-chains (1 per 100) with *N*-hydroxysuccinimide ester cyanine dyes (Z-CyDyes; Z-Cy3, Z-Cy5, Z-Cy2; [21]) according to the manufacturer's protocol (GE Healthcare Bio-Sciences Corp. Piscataway, NY). Briefly, GSH affinity chromatography extracts containing 50 μg protein, were labeled separately on ice with 400 pmol of either Z-Cy3 or Z-Cy5 Cydye DIGE fluors dissolved in DMF. The internal standard, an equimolar mixture of all protein extracts, was labeled with Z-Cy2. Labeling reactions were quenched with 1 μl of 10 mM lysine, held on ice for 10 min, combined appropriately (ex. HS1-Z-Cy3, MHR4-Z-Cy5, and internal standard-Z-Cy2), diluted to a final volume of 450 μl with isoelectric focusing (IEF) buffer (DIGE buffer containing 50 mM DTT and 0.5% (v/v) IPG buffer 3–11 NL [GE Healthcare Bio-Sciences Corp. Piscataway, NY]), and incubated for 1 h at room temperature. IEF and SDS-PAGE followed the methods of Maaty, Selvig, Ryder, Tarlykov, Hilmer, Heinemann, Steffens, Snyder, Ortmann and Movahed [22].

2.3.1. Image acquisition and analysis

Gels were scanned using a Typhoon Trio Imager according to the manufacturer's protocol (GE Healthcare Bio-Sciences Corp. Piscataway, NY 08855) at 100 μm resolution and 640 V for PMT. Images were subjected to an automated difference in-gel analysis using Progenesis SameSpots v 3.0.2 software (Nonlinear Dynamics Ltd. Newcastle, UK). Individual gel spots were co-detected as DIGE image pairs and linked to the corresponding in-gel Z-Cy2 standard to allow between-gel comparisons and statistical analyses. Gels used for protein identification contained 400 μg of protein each and were stained with colloidal coomassie stain [23], destained in 10% acetic acid, and stored at 4 °C in 1% acetic acid until spot excision.

2.3.2. Protein mass determination and analysis

Protein spots of interest were excised, digested with porcine trypsin (Promega Corp. Madison, WI 53711), and eluted as described in Shevchenko, Tomas, Havli, Olsen and Mann [24]. The resulting peptides were subjected to mass analysis performed on Bruker maXis Impact with Dionex 3000 nano-uHPLC controlled with Chromeleon Xpress (2.13 for Hystar). Briefly, samples of 20 μl each were processed via Dionex Ultimate 3000 nano UHPLC, with an Acclaim PepMap100 C18 column (300 μm × 5 mm) used for both trapping and final peptide separation. Chromatography was as follows: solvent consisted of H_2O with 0.1% (v/v) formic acid for channel "A" and acetonitrile for channel "B". Following sample trapping for 2 min at a flow rate of 20 μl min^{-1}, the HPLC valve was switched to elution position. From 0.0 min to 2.5 min, the elution solvent pump composition was held at 5% B. From 2.5 to 20 min, the elution solvent gradient was linearly changed from 5% to 30% B. From 20 to 23 min, the gradient was ramped from 30% to 95% B. From 23 to 28 min, the solvent was held at 95% B, and from 28 min to 30 min the solvent was linearly ramped from 95% B to 7% B. During the entire run, the loading pump solvent was held at 20 μl min^{-1} of 97% water, 3% acetonitrile, and 0.1% formic acid. The mass spectrometer used was a Bruker maXis Impact with CaptiveSpray ESI source with a resolution and accuracy of approximately 40,000 and <5 ppm, respectively. Spectra were collected in positive mode from 200 to 2500 *m/z* at a maximum rate of 2 Hz for both precursor and fragment spectra