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This is the peer reviewed version of the following article: "Keith, Barbara K. , Erin E. Burns, Brian Bothner, Charles C. Carey, Aurelien J. Mazurie, Jonathan K. Hilmer, Sezgi Biyiklioglu, Hikmet Budak, and William E. Dyer. "Intensive herbicide use has selected for constitutively elevated levels of stress-responsive mRNAs and proteins in multiple herbicide-resistant Avena fatua L.." Pest Management Science (August 2017). DOI: 10.1002/ps.4605", which has been published in final form at https://dx.doi.org/10.1002/ps.4605. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

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Intensive herbicide use has selected for constitutively elevated levels of stress-responsive mRNAs and proteins in multiple herbicide-resistant *Avena fatua* L.

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Abstract

BACKGROUND: Intensive use of herbicides has led to the evolution of two multiple herbicide-resistant (MHR) *Avena fatua* (wild oat) populations in Montana that are resistant to members of all selective herbicide families available for *A. fatua* control in US small grain crops. We used transcriptome and proteome surveys to compare constitutive changes in MHR and herbicide-susceptible (HS) plants associated with non-target site resistance.

RESULTS: Compared to HS plants, MHR plants contained constitutively elevated levels of differentially expressed genes (DEGs) with functions in xenobiotic catabolism, stress response, redox maintenance and transcriptional regulation that are similar to abiotic stress-tolerant phenotypes. Proteome comparisons identified similarly elevated proteins including biosynthetic and multifunctional enzymes in MHR plants. Of 25 DEGs validated by RT-qPCR assay, differential regulation of 21 co-segregated with flucarbazone-sodium herbicide resistance in F_3 families, and a subset of 10 of these were induced or repressed in herbicide-treated HS plants.

CONCLUSION: Although the individual and collective contributions of these DEGs and proteins to MHR remain to be determined, our results support the idea that intensive herbicide use has selected for MHR populations with altered, constitutively regulated patterns of gene expression that are similar to those in abiotic stress-tolerant plants.

1 INTRODUCTION

Herbicides are the most widely used tool for weed management worldwide and significantly contribute to increased crop yields and economic returns.[1] Selective graminicides, or herbicides that control weedy grasses without injuring cereal crops, have been used annually for more than 45 years in irrigated malt barley fields in northcentral Montana. The primary target of these herbicide applications is *Avena fatua* (wild oat), a pernicious weed infesting over 40 million hectares in the northern Great Plains alone, where it is one of the most economically costly pests.[2] Malt barley buyers have little or no tolerance for contaminating *A. fatua* seeds, and so regional growers tend to employ rigorous herbicide programs.

Worldwide intensive herbicide use has led to the evolution of herbicide-resistant (HR) weed populations, a rapidly growing problem that causes substantial crop yield losses, increases production costs and poses a significant threat to food security.[1,3] Resistance can be conferred by target site overexpression or mutations that alter herbicide binding, or non-target site resistance (NTSR) alterations like enhanced rates of herbicide metabolism, reduced absorption/translocation, sequestration or changes in abiotic stress defense networks.[4] Of particular concern are multiple herbicide-resistant (MHR) species in many cropping systems,[5] which can be resistant to herbicides not yet on the market.

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Herbicides are obviously designed to control weeds, but they can also merely cause injury, often due to sublethal dosages (improper applications or equipment problems), adverse environmental conditions that reduce efficacy, or delayed applications on older, more tolerant plants.[6] Under these conditions, susceptible plants respond much the same as they do to other abiotic stresses.[7] For example, suites of genes and enzymes of redox maintenance, xenobiotic metabolism and other protective pathways are recruited,[8] likely as a result of signal transduction changes involving abiotic stress-specific[9] and other transcription factors (TFs). Repeated exposure to heat, cold, salt or other abiotic stresses can lead to systemic acquired acclimation (SAA), in which plants evolve enhanced stress resistance due to constitutive, enhanced expression of the genes noted above and others.[10,11] Reactive oxygen species (ROS) and the associated redox regulatory signaling network play a key role in SAA, by transcriptional reprogramming and modulation of metabolism, development and defense responses.[12] Similar to other abiotic stresses, sublethal herbicide exposure induces rapid ROS generation,[13,14] and even though herbicides are designed to inhibit a specific biochemical target, they impact multiple cellular pathways and components.[15,16]

Avena fatua (Poaceae, Pooideae, Aveneae; wild oat), an annual, highly autogamous, allohexaploid monocot weed, is a persistent and highly successful competitor in cereal-growing regions of the world. HR species from the Poaceae family are over-represented[17] among 'the world's worst weed families',[18] and HR *A. fatua* populations probably have a greater worldwide economic impact than those of any other species.[1] We recently described the MHR3 and MHR4 *A. fatua* populations from northcentral Montana 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-sodium (flucarbazone hereafter), the growth inhibitor difenzoquat, the photosystem I inhibitor paraquat (MHR3 only) and the very long chain fatty acid biosynthesis inhibitor triallate, with resistant/susceptible ED_{50} ratios ranging from 1.4 to 57.[19,20] Additional herbicide screens show that MHR3 and MHR4 are resistant to US field use rates of quizalofop-P-ethyl, fluazifop-P-butyl, diclofop-methyl and prosulfocarb (Dyer *et al.*, unpublished). These populations are thus resistant to members of all selective herbicide families available for *A. fatua* control in small grain crops, and pose an ominous and significant challenge for agriculture.

MHR3 and MHR4 resistance to ALS- and ACCase-inhibiting herbicides is not due to known target site mutations, and the cytochrome P450 monooxygenase (P450) inhibitor malathion partially reversed the resistance phenotype for flucarbazone (both populations), imazamethabenz (MHR4), difenzoquat (MHR4) and pinoxaden (MHR3),[19] indicating the involvement of NTSR mechanisms. NTSR evolves gradually under the strong selection imposed by herbicides, appears to be controlled by multiple alleles and is conferred by one or more constitutive and/or induced physiological mechanisms.[21] Recent transcriptome analyses of constitutive changes in HR or MHR populations show that several contigs representing functions in xenobiotic metabolism are more abundant prior to herbicide treatment.[22–24]

Our hypothesis is that constitutive (before herbicide treatment) transcriptome and proteome differences between MHR4 and HS *A. fatua* plants may provide useful insights into NTSR, as well as understanding the similarities with plant responses to other abiotic stresses. We found that the levels of a number of differentially expressed genes (DEGs) and proteins involved in herbicide and abiotic stress response were altered, including those for xenobiotic catabolism, stress response, redox maintenance, biosynthetic processes, transcriptional regulation and multifunctional protein pathways.

2 EXPERIMENTAL METHODS

2.1 Plant material and growth conditions

The MHR4 population was derived from seeds collected in 2006 from an *A. fatua* population not controlled by 60 g a.i. ha^{-1} pinoxaden (1× field rate; Axial, Syngenta Crop Protection Inc., Greensboro, NC, USA; ACCase inhibitor) 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.[19,20] From each generation of 50 plants, all seeds were harvested and a random selection of 50 seeds was used to initiate five additional generations without herbicide selection to homogenize the genetic background. The HS1 population was derived from seeds collected from untreated border plants in an adjacent field, grown for seven generations as described for MHR4 except without herbicide selection, and was subsequently confirmed to be 100% susceptible to the herbicides used in these studies.[19,20] A second susceptible population HS2 used for crossing studies (see below) is the highly inbred non-dormant SH430 line used in seed dormancy research.[25,26] Plants were grown under a 16 h photoperiod of natural sunlight supplemented with mercury vapor lamps (165 μmol m^{-2} s^{-1}) at 25 ± 4 °C in greenhouse soil mix (1:1:1 (v/v/v) Bozeman silt loam:Sunshine mix #12 (Sun Gro Horticulture Inc., Bellevue, WA):perlite) and fertilized weekly with 100 ppm of Jack's Classic 20-20-20 All Purpose fertilizer. For each experiment, plants were grown on the same greenhouse bench until the third leaf was 4–6 cm long (Zadoks' stage 13[27]) and shoots were harvested in mid-morning to minimize potential differences in gene expression due to developmental, environmental and circadian factors.

2.2 Herbicide applications

Three-leaf HS1 and MHR4 seedlings grown as above were treated with either 0.125% v/v nonionic surfactant (X-77, Loveland Products Inc., Loveland, CO) or 30 g a.i. ha^{-1} flucarbazone (1× field rate; Everest™ 70WDG; Arysta LifeScience North America LLC, Cary, NC) plus surfactant in 94 L ha^{-1} of water using a moving nozzle sprayer and returned to the greenhouse. After 6 h, shoot tissue was frozen in liquid nitrogen, total RNA isolated as described in Kern *et al.*[28] and RNA used for qRT-PCR.

2.3 Transcriptome sequencing and analysis

Three replicate HS1 and MHR4 seedlings were grown and treated with surfactant (hereafter referred to as untreated seedlings) as described above, total RNA isolated from shoots after 6 h[28] and contaminating DNA removed with a TURBO DNA-free kit (Thermofisher Scientific, Waltham, MA) according to the manufacturer's instructions. RNA quality for each sample was confirmed (RNA integrity number > 7) (Agilent 2100 Bioanalyser, Agilent, Santa Clara, CA). Six cDNA libraries were generated from 1 μg of total RNA each using TruSeq RNA Sample Preparation v3 (Illumina, San Diego, CA) and random hexamer primers, and each library was bar-coded with unique adaptors to generate 100 bp paired end

reads. Library adaptors were trimmed and quality control checked using FASTQC (v 0.10.1; http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Library construction, high-throughput sequencing with an Illumina HiSeq 2000 (Illumina, San Diego, CA) and quality control were conducted at the University of Wisconsin Biotechnology Center.

De novo transcript assembly was performed at the Montana State University Bioinformatics Core. Reads were digitally normalized using khmer[29] to reduce redundancy from abundant reads prior to *de novo* assembly of each library with Oases.[30] Transcript contigs were clustered and quantified using Corset,[31] and differential gene expression was assessed using edgeR[32] from Corset-derived cluster-level read counts. Annotation of the longest contig within each cluster was performed via blastx against both the uniref90 (release 2014_09[33]) and the Michigan State Rice Genome Annotation Project (release 7[34]) databases, with a cutoff E-value $\leq 10^{-4}$. Clusters were considered differentially expressed if they met the following criteria: fold change (FC) ≥ 1.8, false discovery rate (FDR) corrected *p*-value[35] < 0.1 and fragments per kilobase of transcript per million fragments mapped (FPKM) > 2 for all replicate plants in at least one population.

A. fatua contigs with an E-value $\leq 10^{-4}$ were matched to GO Slim Michigan State Rice addressable *Oryza sativa* (Os) terms and annotated to GO Slim categories (http://rice.plantbiology.msu.edu). The rice genes were labeled for EXPath GO Enrichment analysis[36] as differentially expressed between HS1 and MHR4 if a corresponding *A. fatua* cluster had an FDR-corrected *p*-value <0.1. The universe of all rice genes for each comparison was considered separately for Biological Process (10 755 genes), Molecular Function (10 526 genes) and Cellular Component (9321 genes) GO domains. Fisher exact tests were performed to test for enrichment of each GO Slim category among the genes labeled differentially expressed, and the resulting *p*-value corrected by FDR.

A separate analysis independent of the RNA-Seq transcriptome assembly was conducted using rice or Brachypodium GenBank accessions with strong homology (E-value $\leq 10^{-25}$) to Michigan State University Rice Genome annotations of differentially expressed RNA-Seq contigs (supporting information, file 2). This secondary assessment of differential gene expression used ArrayStar QSeq software (v 5.0; DNASTAR Inc., Madison, WI) to map RNA-Seq fastq data to each of the NCBI accessions. Contigs with FC ≥ 1.6 and FPKM ≥ 2 for all replicate plants in at least one biotype were considered differentially expressed.

2.4 RT-qPCR assays

HS1 and MHR4 seedlings were grown in the greenhouse and shoots harvested at 0 h and 6 h after treatment with surfactant or herbicide as described above. Total RNA was isolated and DNase treated as described above, and reversed transcribed with SuperScript^ IV Reverse Transcriptase (Invitrogen, Thermofisher Scientific, Waltham, MA) following the manufacturer's protocol.

RT-qPCR primer pairs (supporting information, file 4) were designed using PrimerQuest software (Integrated DNA Technologies; Coralville, IA) for 25 DEGs, chosen based on GO Slim assignments, FPKM values, our preliminary enzyme and expression assays (Burns *et al.*, submitted) and knowledge about plant responses to herbicides. RT-qPCR reactions contained 150 ng cDNA, 1× DyNAmo™ Flash SYBR^ Green master mix (Thermo Fisher Scientific, Waltham, MA) and 200 nM each primer in a total volume of 20 μL using a Rotor-Gene 3000 (Qiagen, Germantown, MD) real-time analyzer. Cycle parameters were: hold 95 °C for 7 min, 40 cycles of 95 °C for 10 s, 63 °C for 30 s and 72 °C for 30 s, followed by a final extension cycle of 72 °C for 5 min. All amplicons spanned the stop codon, and RT-qPCR reactions were subjected to melt curve analyses and reaction efficiencies according to MIQE guidelines.[37]

RT-qPCR results from duplicate experimental reactions from two or three biological replicates were normalized against two validated reference genes with stable expression: the wheat cell division control protein from the ATPase AAA superfamily (Ta54227; GenBank accession EU267938[38]) and an *A. fatua* alpha glucan phosphorylase gene (Cluster 5253; supporting information, file 2) as identified through NormFinder.[39] Threshold-cycle (*C*T) values were determined for each reaction using Rotor-Gene 6 software (version 6.1; Qiagen, Germantown, MD) and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.[40] Control reactions to test for DNA contamination contained DNase-treated RNA but not reverse transcriptase.

2.5 F_3 families and co-segregation analysis

Reciprocal crosses between homozygous MHR3, MHR4 and HS2 plants were performed as described by Brown[41] for *A. sativa* cross-pollination (Burns *et al.*, submitted). HS2 was used as the susceptible parent since it has never been exposed to herbicide treatment.[25,26] Ten seeds each from homozygous resistant and susceptible F_2 families were grown in the greenhouse to Zadoks' stage 23[27] and vegetatively cloned by separating into three tillers each, which were replanted and returned to the greenhouse for 7 days. Shoot tissue from one vegetative clone from each family was harvested, frozen in liquid nitrogen and stored at −80 °C until injury screenings were completed. An additional clone from each family was treated with 30 g a.i. ha^{-1} flucarbazone as described above and rated after 21 days using a visual injury scale of 0 = no injury to 4 = complete kill to determine HS (injury score 3.5–4.0) and HR (injury score = 0–0.5) phenotypes. Two F_3 plants from each F_2 family with resistant and susceptible phenotypes were selected and used for RNA isolation and RT-qPCR assays as described above. Additional screens of resistant F_4 progeny confirmed no segregation of the resistance phenotype (data not shown).

2.6 Proteome analysis

Shoot tissue (200 mg) from four replicate three-leaf HS1 and MHR4 plants grown as described above was ground under liquid nitrogen and suspended in ice-cold extraction buffer 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 filtered through Miracloth (EMD Millipore, Merck, Darmstadt, Germany) and filtrates were centrifuged at 21 380 × *g* for 10 min at 4 °C. Proteins were concentrated by precipitation with four volumes of ice-cold acetone containing 10% TCA at −80 °C overnight. Two additional 100% acetone precipitations were performed for 3 h each at −80 °C, and proteins were resuspended in DIGE buffer (30 mM Tris, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS, 1× protease inhibitor/nuclease mix (GE Healthcare Life Sciences, Pittsburgh, PA) and 0.1% (w/v) bromophenol blue) to a final concentration of 2 mg mL^{-1}. Protein concentrations were determined[42] using bovine serum albumin fraction V as standard.

Two-dimensional fluorescence difference gel electrophoresis (2-D 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 and Z-Cy2,[43] according to the manufacturer's protocol (GE Healthcare Bio-Sciences Corp., Piscataway, NY)). Briefly, extracts containing 50 μg of protein were labeled separately on ice with 400 pmol of either Z-Cy3 or Z-Cy5 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 (i.e. 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 *et al.*[44]

2.7 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) at 100 µm resolution at 640 V for the photomultiplier tube. Gel spots were co-detected as DIGE image pairs and between-gel comparisons were performed using Progenesis SameSpots software v 3.0.2 (Nonlinear Dynamics Ltd, Newcastle, UK) utilizing the in-gel Z-Cy2 standard for each pair. Gels used for protein identification each contained 400 µg of protein and were stained with colloidal Coomassie stain,[45] destained in 10% (v/v) acetic acid and stored at 4 °C in 1% (v/v) acetic acid until spot excision.

2.8 Protein mass determinations and analysis

After electrophoresis, all significantly differential protein spots (*p*-value <0.11 identified by SameSpots) and RuBisCO (as an internal standard) were excised, digested with porcine trypsin (Promega Corp., Madison, WI) and eluted as described in Shevchenko *et al.*[46] The resulting peptides were subjected to peptide mass fingerprinting using a Bruker maXis Impact with Dionex 3000 nano-UHPLC controlled with ChemStation LC 3D (Rev A.10.02) following the methods of Maaty *et al.*[44]

Protein identification followed the methods outlined in Mason *et al.*,[47] initiated by generating a custom protein sequence file (.FASTA) from the *Oryza sativa subsp. japonica* (retrieved 2016; http://www.UniProt.org; containing 121 989 entries) and *Brachypodium distachyon* (retrieved 2016; http://www.UniProt.org; containing 50 507 entries) databases. Sequences for potential contaminants human keratin (retrieved 2016; http://www.UniProt.org; containing 49 entries) and porcine trypsin (retrieved 2016; http://www.UniProt.org; containing 1 entry) were added to create a final target-decoy library. The library was queried against data files using the following search parameters: up to two missed cleavages allowed; precursor charges +2, +3, +4; precursor ion mass tolerance 30 ppm; and fragmentation mass tolerance of 0.5 Da. Post-translational modifications were defined as: oxidation of M; acetylation of N-terminus; and carboxylation of C-terminus. Two or more significant peptides with an FDR corrected *p*-value ≤1.0 (PeptideShaker software[48]) were required for annotation of each protein from the PaxDb database[49] as accessed through UniProtKB and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

3 RESULTS

Pinoxaden-resistant *A. fatua* seeds from field collections were subjected to seven generations of recurrent group selection and subsequently determined to be resistant to at least 10 herbicides from five mode of action families,[19,20] including flucarbazone, a sulfonylaminotriazolinone ALS-inhibitor herbicide for which little is known about NTSR mechanisms.[50] Flucarbazone was therefore used as a test herbicide for the MHR4 and HS1 RT-qPCR assays and co-segregation studies reported below.

3.1 Transcriptome analysis

RNA-Seq transcriptome sequencing of *A. fatua* yielded a total of 539 million reads, resulting in an average of 58 and 56 million normalized trimmed reads for HS1 and MHR4 plants, respectively (Table 1). A total of 2.2 and 2.15 million contigs were assembled from HS1 and MHR4 reads, respectively, with an average contig length of 1200 bp for both biotypes. Corset analysis resulted in 23 112 unique clusters and 21 197 were annotated to an expect-value (E-value hereafter) ≤ 10^{-4}. Of the 584 differentially expressed clusters meeting the criteria listed in Section 2, 324 and 260 were present at lower and higher levels, respectively, in MHR4 as compared to HS1 plants.

The resulting contigs corresponded to 11 868 GO Slim Michigan State Rice addressable Os terms that were annotated to 97 GO Slim categories, of which 45 were in Biological Process, 26 in Molecular Function and 26 in Cellular Component GO domains (Appendix S1). Within these domains, eight, three and eight GO terms, respectively, were over-represented (FDR corrected *p*-value <0.1)[35] and contained a total of 453 unique differentially expressed contigs (DECs) between MHR4 and HS1 plants (Table 2). Expression levels of DECs in MHR4 plants were higher for 51, 52 and 43% of the totals in the GO Biological Process, Molecular Function and Cellular Component domains, respectively. Within the Biological Process domain, the Response to Stress term had the lowest FDR corrected *p*-value (1.2^{-8}) and contained 1335 contigs, comprised of 119 DECs and 1216 similarly expressed contigs. The 119 DECs assigned to the Response to Stress term represent 26% of the 453 unique DECs, while the 1216 of 11 415 similarly expressed contigs represent only 11%, confirming that DECs for stress response are enriched.

DECs assigned to the Response to Stress GO term were further analyzed by EXPath GO Enrichment analysis[36] (Fig. 1). Of terms containing >10% of the total assignments, those with notably higher expression in MHR4 plants include: transport, small-molecule metabolic process, response to sucrose, response to salt stress, response to oxidative stress, oxidoreductase activity, oxidation – reduction process, immune system process, hydrolase activity, DNA binding, cellular amino acid metabolic process and catabolic process (Fig. 1). Terms with lower expression in MHR4 plants include: transporter activity, response to heat, reproduction, protein folding, protein binding, kinase activity, isomerase activity, cellular protein modification process and ATP binding. Overall, GO Slim assignments indicate that the constitutive physiological states of MHR4 and HS1 plants are markedly different, with MHR4 exhibiting heightened responses to several stresses and elevated stress-related processes. The reduced protein folding and protein binding terms in MHR4 are also noteworthy, as discussed below.

To independently verify differential expression, we used ArrayStar QSeq analysis of 390 unique GenBank accession numbers homologous to *A. fatua* RNA-Seq DECs with a Michigan State Rice Genome E-value <10^{-25}. Of the 129 DECs with FPKM >2 and FC > 1.6, four did not match the RNA-Seq expression patterns and were discarded (Appendix S2).

Based on GO assignments and the criteria listed in Section 2, 25 DEGs were chosen from this dataset for RT-qPCR validation assays (Table 3). Overall, 18 and 7 of the validated DEGs were detected at higher and lower levels in MHR4 plants, respectively, as compared to HS1 plants, and individual DEGs are discussed below. In addition to determining expression levels in untreated MHR4 and HS1 plants, their co-segregation with flucarbazone resistance

Table 1. Results from RNA-Seq analysis of herbicide-susceptible (HS1) and multiple herbicide-resistant (MHR4) *A. fatua*

	HS1			MHR4		
	Plant 1	Plant 2	Plant 3	Plant 1	Plant 2	Plant 3
Sequencing						
Total raw reads (paired ends)	1.15×10^8	6.59×10^7	9.32×10^7	8.83×10^7	9.20×10^7	8.52×10^7
Total bp sequenced	1.15×10^9	7.38×10^8	7.65×10^8	8.16×10^8	9.50×10^8	8.93×10^8
Reads trimmed (paired ends)	1.05×10^8	5.89×10^7	8.23×10^7	7.75×10^7	8.41×10^7	7.63×10^7
Read length trimmed	86	86	86	86	86	86
Phred quality score (Q) after trimming	Q37	Q37	Q37	Q37	Q37	Q37
Total reads normalized/trimmed (paired ends)	2.34×10^7	1.59×10^7	1.82×10^7	1.77×10^7	1.93×10^7	1.88×10^7
Total bases normalized/trimmed (paired ends)	1.87×10^9	1.25×10^9	1.43×10^9	1.39×10^9	1.54×10^9	1.47×10^9
Median sequence length (nt)	79.71	80	78.67	78.55	80	78.45
Total % duplicate	41.72	32.53	37.14	33.49	38.23	34.39
Assembly						
Number contigs	9.11×10^5	6.17×10^5	6.69×10^5	6.67×10^5	7.57×10^5	7.24×10^5
Median contig size	1.26×10^3	1.20×10^3	1.14×10^3	1.22×10^3	1.26×10^3	1.23×10^3
N50 contig size	1846	1750	1660	1777	1840	1827

Table 2. GO assignments of similarly and differentially expressed contigs (DECs) in MHR4 *A. fatua*

GO term				Contigs in GO term				
GO domain	ID	Description	FDR corrected *p*-value	Total contigs (no.)	Similarly expressed contigs (no.)	DECs (no.)	DECs reduced in MHR4 (%)	DECs elevated in MHR4 (%)
Biological process	GO0009987	Cellular process	6.2×10^{-2}	3442	3249	193	51	49
	GO0008152	Metabolic process	2.3×10^{-4}	3131	2933	198	46	54
	GO0006950	Response to stress	1.2×10^{-8}	1335	1216	119	56	44
	GO0009628	Response to abiotic stimulus	2.1×10^{-5}	958	876	82	56	44
	GO0009056	Catabolic process	5.9×10^{-2}	739	688	51	39	61
	GO0009607	Response to biotic stimulus	8.6×10^{-3}	381	347	34	59	41
	GO0006091	Generation of metabolites and energy	1.3×10^{-2}	174	155	19	63	37
	GO0015979	Photosynthesis	4.8×10^{-3}	128	111	17	71	29
Molecular function	GO0003824	Catalytic activity	4.9×10^{-3}	1655	1545	110	46	54
	GO0016787	Hydrolase activity	4.3×10^{-2}	1305	1224	81	42	58
	GO0005198	Structural molecule activity	4.3×10^{-2}	275	251	24	71	29
Cellular component	GO0009536	Plastid	1.5×10^{-8}	1920	1758	162	59	41
	GO0016020	Membrane	1.3×10^{-2}	1799	1676	123	60	40
	GO0005829	Cytosol	1.9×10^{-2}	1299	1207	92	60	40
	GO0005773	Vacuole	5.9×10^{-2}	614	567	47	62	38
	GO0009579	Thylakoid	2.8×10^{-6}	329	285	44	66	34
	GO0005840	Ribosome	4.8×10^{-3}	240	213	27	70	30
	GO0005618	Cell wall	7.0×10^{-2}	330	302	28	61	39
	GO0005576	Extracellular region	3.2×10^{-5}	191	162	29	48	52

in F_3 families and their response in HS1 plants after herbicide treatment were determined.

3.2 Proteome analysis

To investigate changes at the protein level, 2-D DIGE[51] of abundant soluble proteins resolved 912 (HS1) and 917 (MHR4) individual protein spots (Fig. 2). Statistical analysis showed that 34 were significantly different between groups, and in-gel trypsin digestion followed by LC–MS/MS identified 28 unique proteins, with 6 spots failing to produce unambiguous results (Table 4). Overall, levels of 19 proteins were constitutively higher in MHR4 than HS1 plants, and 9 proteins were lower.

4 DISCUSSION

This is the first study combining transcriptome and proteome analyses to investigate the changes associated with NTSR in any MHR

Table 3. DEGs from multiple herbicide-resistant (MHR4) and herbicide-susceptible (HS1) *A. fatua*

Functional category	RNA-Seq					RT-qPCR FC		
	Contig	Annotation	E-value	FC	FDR corrected *p*-value	MHRc/HSc	HSt/HSc	F3 fr/ F3 fs
Xenobiotic response/catabolic processes	5954.7	CYP81A6	1.2×10^{-80}	1.9	0.064	3.4 (0.9)	1	2.2 (0.2)
	6502	CYP94C1	0	−3.4	0.034	−2.5 (0.3)	−1.9 (0.1)	1
	7493.3	GST lambda IN2-1	9.7×10^{-102}	2.2	< 0.0001	2.0 (0.6)	1	3.3 (0.2)
	1351.5	GST phi AmGSTF1	9.3×10^{-134}	> 10	< 0.0001	> 10	1	> 10
	654	GST tau Cla-47	6.7×10^{-87}	4.0	0.0002	5.4 (1.0)	1	> 10
	358	GST tau U6	1.1×10^{-92}	2.9	< 0.0001	7.3 (1.5)	1	8.4 (2.5)
	3532	UDP-glucosyl-transferase	8.2×10^{-158}	3.6	0.098	> 10	3.8 (1.5)	6.6 (0.6)
	5629.48	Glycosyl-transferase	0	2.1	0.0005	2.9 (0.6)	1	2.9 (0.8)
	1438	ABC transporter C subfamily	0	2.7	< 0.0001	2.3 (0.1)	1	3.5 (0.6)
	4170	ABC transporter C subfamily	0	2.1	< 0.0001	2.6 (0.9)	2 (0.5)	2.9 (0.1)
	1335.2	MATE transporter	0	2.1	0.043	2.1 (0.2)	1	1.9 (0.1)
Stress response/ redox maintenance	5629.9005	Acyl-coA dehydrogenase	0	1.8	0.074	3.2 (0.6)	2.1 (0.2)	2.0 (0.1)
	3435.1	Superoxide dismutase, chloroplastic	1×10^{-152}	1.9	0.01	> 10	1	> 10
	2123	Ornithine δ-amino-transferase	0	5.8	< 0.0001	6.5 (2.0)	5.0 (1.2)	5.3 (2.1)
	5629.3793	sHsp24	2×10^{-87}	> −10	< 0.0001	−4.8 (0.9)	1	−8.4 (0.9)
	5629.2548	Hsp90	0	−3.0	< 0.0001	−3.4 (0.5)	1	−2.4 (0.6)
	7001.7	sHsp17	3.8×10^{-44}	−7.6	0.007	−4.9 (2.3)	3.8 (0.4)	−2.6 (0.3)
	4994	Peptidylprolyl isomerase FKBP domain	0	−5.3	< 0.0001	−3.5 (0.3)	2.3 (0.1)	−5.6 (0.4)
	4033	HAD phosphatase	1.1×10^{-54}	−5.8	0.026	−10 (5.6)	1	1
Transcription factors/regulators	2018.3	SNF2 ATPase	0	3.4	0.056	6.3 (1.2)	2.1 (0.1)	5.6 (0.3)
	5629.5756	JMJ18-like JmjC demethylase	0	2.1	0.0002	3.4 (0.4)	1	1
	5629.7288	Utp12-like WD40	0	−2.2	0.002	−2.1 (0.1)	1	1
	5629.4394	NBS-LRR (RPP13-like)	0	> 10	< 0.0001	> 10	1	> 10
Flavonoid pathway	3574	Anthocyanidin 3-*O*-glucosyl transferase	0	> 10	< 0.0001	4.9 (2.0)	−2.4 (0.4)	3.7 (1.0)
	473.1	Anthocyanin bHLH-Myc TF	6.9×10^{-110}	> 10	< 0.0001	3.4 (1.1)	−4.6 (0.3)	3.5 (1.4)

Fold change (FC) values from RNA-Seq analysis and RT-qPCR validation assays of expression levels in untreated MHR and HS plants (MHRc/HSc), flucarbazone-treated HS plants (HSt/HSc), where t = treated and c = surfactant only control, and in segregating F_3 plants with flucarbazone-resistant (fr) and flucarbazone-sensitive (fs) phenotypes. FC of 1 indicates no change, positive and negative FC values indicate higher and lower levels in MHR4 compared to HS1 plants, respectively.

plant species. Peng[52] used both techniques to show that altered translocation patterns were associated with glyphosate resistance in *Conyza canadensis*, and others have used the combination to examine the response of susceptible bacteria[53,54] or plants[55,56] to various herbicides. Several laboratories have investigated herbicide resistance using either transcriptional profiling or proteome analysis alone,[11,57] with a general focus on changes induced after herbicide treatment.

Eukaryotic organisms have adopted a number of strategies to deal with abiotic stress,[58] and Kültz[59] identified 300 proteins shared among the stress responses of photosynthetic microorganisms, plants and invertebrates. Naturally stress-tolerant plant species and varieties contain elevated levels of TFs, ROS scavenging enzymes, glutathione *S*-transferases (GSTs) and other protective enzymes[60] like those documented here for MHR4 *A. fatua*. In general, plant stress response can be considered in five phases: alarm, acclimation, resistance and exhaustion (if stress is too severe) or recovery to a new homeostasis.[11] We suggest that the constitutive transcriptome and proteome changes described below provide insights into the new homeostasis exhibited by MHR *A. fatua*, which is maintained in the absence of herbicide stress.

In the following sections, the DEGs and proteins from Tables 3 and 4, respectively, are discussed in five functional categories: xenobiotic response/catabolic processes, stress response/redox maintenance, biosynthetic and flavonoid pathways, multifunctional proteins and TFs/regulators.

4.1 Xenobiotic response/catabolic processes

Numerous P450s are associated with resistance to herbicides, including CYP76[61] and CYP81A6.[62] In *A. fatua*, five DEGs annotated as P450s met our RNA-Seq criteria for differential expression between MHR4 and HS1 plants, with expression differences validated by RT-qPCR for 5954.7 and 6502 (Table 3). DEG 5954.7

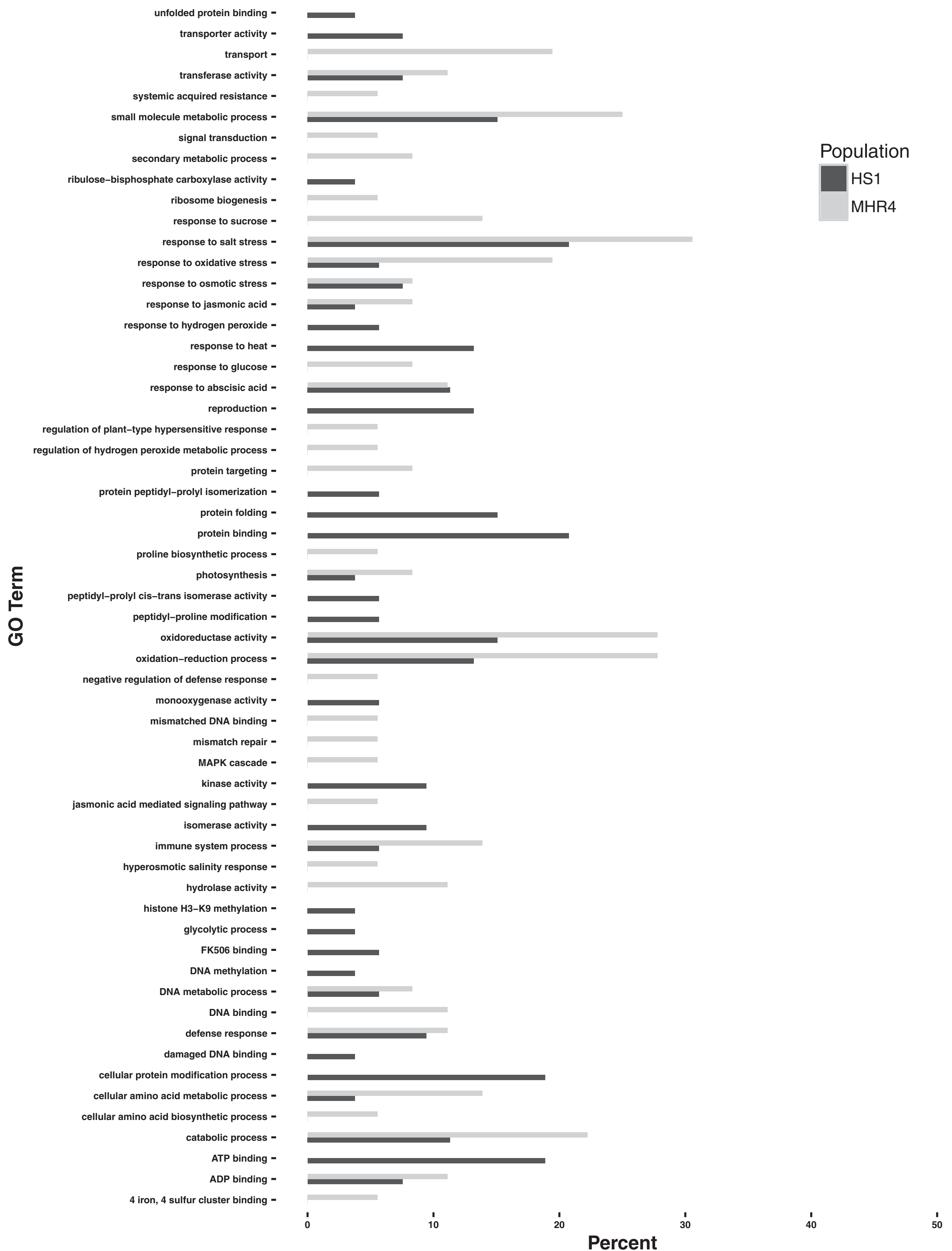

Figure 1. GO assignments of differentially expressed contigs (DECs) from HS1 and MHR4 *A. fatua*.

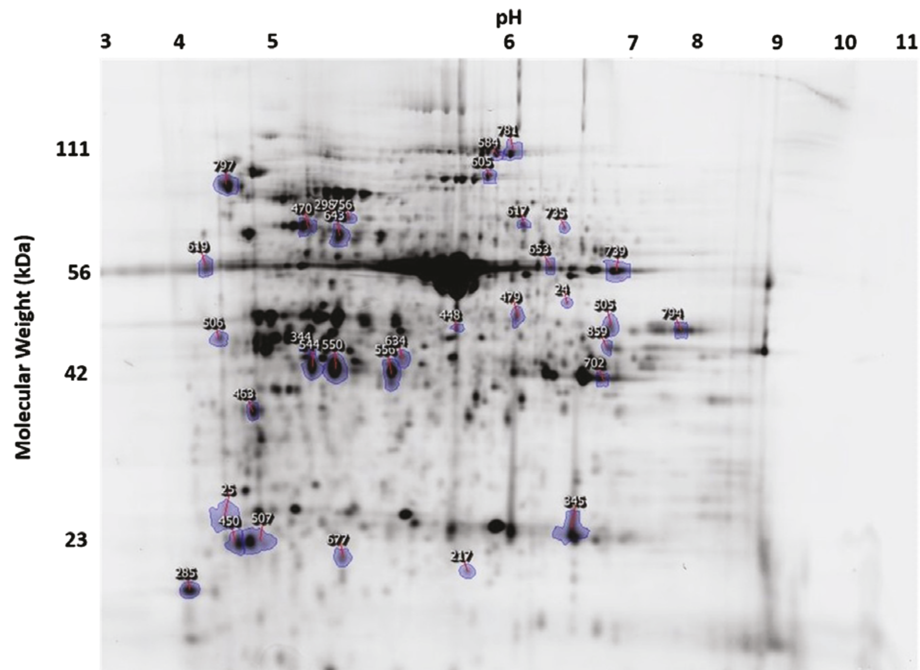

Figure 2. DIGE gel of detectable proteins from MHR4 *A. fatua* with numbered, differentially expressed spots.

(CYP81A6), shown to confer resistance to bentazon in transgenic Arabidopsis,[63] was constitutively elevated in MHR4 plants and its differential regulation co-segregated with flucarbazone resistance in F_3 families. DIGE analysis showed that MHR4 plants contained higher levels of spot 25 (*O. sativa* 76C4 P450), a wound-inducible P450[64] (Table 4). In contrast, DEG 6502 (CYP94C1), involved in jasmonate response and salt stress alleviation,[65] was constitutively lower in MHR4 than HS1 plants. These mixed results, along with the fact that malathion only partially reversed the MHR phenotype,[19] indicate that P450-mediated herbicide metabolism may not play a central role in MHR *A. fatua*, in contrast to results from other species.[22,66]

Enhanced activities of phase II enzymes like GSTs and glucosyltransferases (GTs) are associated with herbicide resistance in several weedy species.[67–69] Recent transcriptome analyses of HR and MHR populations have identified a number of glutathione-related transcripts associated with resistance.[22,23] In *A. fatua*, DEGs 654 (Cla-47 tau GST),[70] 358 (tau GST-U6),[71] 7493.3 (lambda IN2-1 GST)[72] and 1351.5 (AmGSTF1 phi GST)[73] were all present at higher constitutive levels in MHR4 plants and their differential regulation co-segregated with flucarbazone resistance in F_3 families (Table 3). DEG 1351.5 has 87% deduced amino acid identity to *Alopecurus myosuroides* AmGSTF1, which confers resistance to alachlor, atrazine and chlorotoluron in *A. myosuroides* and transgenic Arabidopsis.[73] Identification of a putative AmGSTF1 ortholog at elevated levels in MHR4 *A. fatua* is intriguing, given the markedly different herbicide resistance spectra in the two species. However, in contrast to results from *A. myosuroides*,[74] none of the *A. fatua* GST DEGs were induced or attenuated in HS1 plants after herbicide treatment (Table 3). At the protein level, it is perhaps surprising that we did not identify any differential GSTs, since they can constitute up to 2% of the total protein in cereal plant shoots.[75]

GTs are involved in phase II metabolism of atrazine[76] and other herbicides,[77] but they also have key roles in conferring tolerance to other abiotic stresses like cold, drought and salt by modulating anthocyanin accumulation.[78,79] In this study, three DEGs (3532, 5629.48 and 3574) were annotated as GTs, all of which were constitutively elevated in MHR4 plants and their differential regulation co-segregated with flucarbazone resistance in F_3 families (Table 3).

The eight ABC transporter subfamilies in plants have diverse roles in physiology, especially involving responses to abiotic and biotic stresses .[78,80] Specifically members of the C subfamily play a critical role in herbicide detoxification[81–83] and have been invoked in conferring glyphosate resistance in *Conyza Canadensis*.[84,85] In this study we identified two DEGs (1438 and 4170) annotated as C subfamily ABC transporters. In addition, DEG 1335.2 was annotated as a functionally related MATE transporter, responsible for anthocyanin transport into the vacuole and efflux of numerous xenobiotics.[86] Levels of all three transporters were constitutively elevated in MHR4 plants and their differential regulation co-segregated with flucarbazone resistance in F_3 families.

4.2 Redox maintenance/stress response

As noted above, abiotic stresses including herbicides cause perturbations in redox homeostasis including rapid ROS generation. For example, four of the five herbicides/families in the *A. fatua* MHR resistance spectrum, i.e. paraquat,[87] difenzoquat,[88] ALS inhibitors[89] and ACCase inhibitors,[90] are known to induce ROS production in susceptible species. Thus it may be expected that MHR4 plants exhibit enhanced capacity for redox maintenance, given that they are resistant to at least eleven herbicides from five different mode of action families. In this regard, three DEGs annotated as redox-related enzymes were constitutively elevated in MHR4 plants and validated by RT-qPCR assay (Table 3). The differential regulation of DEG 5629.9005 (acyl-coA dehydrogenase), involved in mitochondrial lipid β-oxidation repair in response to heat stress in *C. elegans*,[91] DEG 3435.1 (chloroplast Fe-superoxide dismutase), a stress-generated ROS detoxifier,[92] and DEG 2123 (ornithine-δ-aminotransferase), a proline biosynthetic enzyme that enhances ROS-scavenging capacity and confers drought and oxidative stress tolerance,[93] co-segregated with flucarbazone resistance in F_3 families.

Table 4. Differentially expressed proteins from HS1 and MHR4 *A. fatua*

Functional group	Spot no.	No. of peptides	Uniprot no.	*Oryza sativa* (Os) no.	Annotation	FC	*p*-value
Stress response/redox maintenance	24	3	I1H4C6	Os07g0108300	Glutamate-glyoxylate aminotransferase	1.6	0.002
	25	2	Q33AN2	Os10g09100	Cytochrome P450	1.6	0.027
	605	2	Q0JEU8	Os04g0201900	Protein kinase	1.5	0.102
	617	2	Q0JEU6	Os04g12580	D-Mannose binding lectin	1.5	0.088
	635	2	Q9FTT4	Os01g0239100	Hsp/DnaJ family protein	1.5	0.105
	797	3	I1HKJ4	Os05g23740	Similar to chloroplast hsp70	−1.5	0.005
Biosynthetic pathways	298	2	Q53M10	Os11g0244100	Transferase family protein	1.5	0.063
	345	2	I1HTF8	Os01g0851700	Cytosolic starch phosphorylase	1.6	0.103
	479	2	Q5KQH5	Os05g0482700	2,3-Bisphospholycerate independent phosphoglycerate mutase	1.6	0.097
	506	16	I1HTG2	Os04g0234600	Sedoheptulose-1,7-bisphosphatase	1.7	0.108
	756	5	I1H1V5	Os06g0133800	Transketolase, chloroplastic	1.6	0.061
	781	7	I1HQW3	Os06g0611900	Glycine dehydrogenase	1.6	0.098
Transcription factors/regulators	450	2	B5AEK5	Os12g09250	bZIP TF	1.7	0.078
	463	2	B9FGL5	Os05g03550	Myb/SANT-like DNA-binding domain	−1.6	0.102
	470	10	I1HIC9	Os05g0481400	TF B3 family protein	1.6	0.102
	505	2	Q5JLR7	Os01g0758200	Similar to Dof2	1.5	0.101
	507	2	Q10N77	Os03g0279500	Similar to HLH DNA-binding domain	−1.7	0.103
	739	2	I1HCP3	Os01g0180600	DNA mismatch repair: MSH6	1.5	0.078
	794	2	I1I810	Os08g37810	Myb/SANT-like DNA-binding domain	1.5	0.098
Multifunctional proteins	448	8	I1IN47	Os11g0171300	Fructose-bisphosphate aldolase	−1.5	0.042
	544	5	I1IN47	Os11g0171300	Fructose-bisphosphate aldolase	−1.6	0.061
	550	12	I1IN47	Os11g0171300	Fructose-bisphosphate aldolase	1.5	0.101
	556	9	Q7X8A1	Os04g0459500	Glyceraldehyde-3-phosphate dehydrogenase	1.5	0.102
	735	2	I1HA60	Os03g03720	Glyceraldehyde-3-phosphate dehydrogenase	−1.6	0.099
Various	217	4	I1H3Y8	Os07g0141400	Photosystem II oxygen-evolving enhancer protein 2	−1.8	0.089
	285	2	Q10PC0	Os03g13660	Unknown protein	−1.7	0.101
	344	2	B7F845	Os08g0558800	Ribosomal protein L1p/L10e family	−1.6	0.070
	677	2	Q2QXU3	Os12g04880	Retroviral aspartyl protease	1.7	0.102

Positive and negative fold change (FC) values indicate higher and lower levels in MHR4 compared to HS1 plants, respectively.

Heat shock proteins (Hsps) and the diverse families of small Hsps in plants are known to be induced or repressed in response to biotic and abiotic stresses.[94,95] In addition to their roles as molecular chaperones that prevent protein misfolding, some Hsps regulate TFs for a diverse set of genes,[96] and several Hsps are involved in adaptation of *Saccharomyces cerevisiae* to the auxinic herbicide 2,4-D.[97] In *A. fatua*, we observed constitutive reduction of three Hsp DEGs (5629.3793, 5629.2548 and 7001.7) and one Hsp protein (spot 797) in MHR4 *A. fatua* (Tables 3 and 4). The functionally related DEG 4994 (cyclophilin-like peptidylprolyl isomerase), which catalyzes a rate-limiting step in protein folding,[98] was also constitutively reduced in MHR4 plants. Reduced levels of all four features co-segregated with flucarbazone resistance in F_3 families. Only one Hsp protein (spot 635), annotated as a member of the Hsp/DnaJ family, was constitutively elevated in MHR4 plants.

The reduced constitutive profile of Hsps documented here for MHR4 *A. fatua* has also been reported in other stress-tolerant biotypes. For example, a comparison of natural *Chenopodium album* biotypes from thermally variable environments showed that biotypes adapted to more stressful environments contained lower levels of certain Hsps than those from moderate habitats, both constitutively and in response to thermal stress.[99] Similar results were reported for *Drosophila*[100] and Arabidopsis,[101] suggesting that this is an evolutionarily conserved strategy for heat stress acclimation, proceeding through an as yet unknown mechanism. Our results indicate that a very different stress, i.e. herbicide damage, has caused *A. fatua* plants to adopt a similar strategy during herbicide selection over more than 45 years, leading to a heritable downregulation of certain Hsps.

Other stress-responsive DEGs and proteins detected in MHR4 plants include DEG 4033 (HAD hydrolase), involved in cellular detoxification,[102] which was reduced in untreated MHR4 plants and flucarbazone-treated HS1 plants (Table 3). In contrast, three stress-responsive proteins were constitutively elevated in MHR4 plants, including spot 24 (glutamate-glyoxylate aminotransferase), a key photorespiratory enzyme induced by abiotic

stress,[103] spot 605 (abiotic stress-responsive protein kinase)[104] and spot 617 (D-mannose binding lectin) involved in resistance to pests[105] and other stressors[106] (Table 4). GO Slim enrichment assignments (Fig. 1) related to the redox maintenance/stress response functional category aligned with RT-qPCR results, in that GO terms for response to oxidative stress, oxidoreductase activity, oxidation – reduction process, hydrolase activity and catabolic process were enriched in MHR4 plants, while the term for response to heat was reduced.

4.3 Biosynthetic processes

Naturally stress-tolerant species and biotypes can exhibit constitutively elevated transcripts and enzymes of core biosynthetic processes,[11] perhaps as a result of resource reallocation needs for adaptive mechanisms.[107] In this regard, six such enzymes involved in glycolysis, the Calvin cycle, C-2 glycolate pathway and related biosynthetic processes were identified at constitutively elevated levels in MHR4 plants from DIGE gels (Table 4). These include spots 298 (transferase family protein), 345 (starch phosphorylase), 479 (2,3-bisphosphoglycerate-independent phosphoglycerate mutase), a salt stress-induced glycolytic enzyme,[108] spot 506 (sedoheptulose-1,7-bisphosphatase), which confers enhanced CO_2 assimilation when overexpressed,[109] 756 (a chloroplast transketolase) and 781 (glycine dehydrogenase), which is upregulated in response to salt stress.[110]

4.4 TFs/regulators

Plant responses to abiotic stresses are initiated by the modulation of a number of genes that confer protective and adaptive functions. These changes are mediated by TFs that are responsive to specific[9] and overlapping[111] environmental stresses, such as MYB, NAC and basic leucine zipper (bZIP) TFs. In *A. fatua*, four validated DEGs were annotated as TFs or related proteins, three of which were constitutively elevated in MHR4 plants (Table 3), and elevated expression of DEGs 2018.3 (SNF2 ATPase TF)[112] and 5629.4394 (nucleotide binding site – leucine rich repeat (NBS-LRR) gene) co-segregated with flucarbazone resistance in F_3 families. NBS-LRR genes represent the majority of R genes in plants and confer pathogen resistance[113] and drought and salt tolerance.[114] Levels of DEG 5629.5756 (JmjC histone demethylase), a key regulator of differentiation, growth and stress-responsive genes,[115] were also constitutively elevated. In contrast, the TF DEG 5629.7288 (WD40 protein)[116] was detected at lower levels in MHR4 plants.

At the protein level, five of seven spots identified as TFs or related proteins were constitutively elevated in MHR4 plants. Spot 505 was identified as a DNA-binding with one finger (Dof2) domain protein, a plant-specific TF involved in diverse physiological processes.[117] Spots 450 and 739 were identified as a bZIP TF and a DNA mismatch repair protein MSH6, respectively, proteins widely involved in developmental and abiotic stress responses.[118,119] Of two spots (463 and 794) containing the MYB-related SANT domain,[120] spot 794 was constitutively elevated in MHR4 plants, while spot 463 was reduced. Overall, the presence of eight constitutively elevated TFs or related proteins in MHR4 *A. fatua* plants supports the idea that these plants possess altered aspects of transcriptional programming. These changes are similar to those in abiotic stress-tolerant plants,[11] and we suggest that they represent promising candidate genes for further investigations of NTSR mechanisms.

4.5 Flavonoid/anthocyanin pathways

Beyond their use as pigments and signaling molecules, flavonoids and especially anthocyanins are well known to protect against a number of environmental stresses.[121] DEGs for four of five proteins in the flavonoid/anthocyanin biosynthetic pathway were constitutively elevated in MHR4 plants and their differential regulation co-segregated with flucarbazone resistance in F_3 families (Table 3). As noted above, three DEGs (3532, 5629.48 and 3574) were annotated as GTs, of which DEG 3574 (anthocyanidin 3-*O*-glucosyltransferase) and DEG 473.1 (maize R bHLH-Myc TF) are a key enzyme and regulator of the anthocyanin pathway, respectively.[122–124] FC values for these two DEGs were among the highest reported in Table 3, and surprisingly both genes were attenuated in flucarbazone-treated HS1 plants. Thus, constitutively increased DEG levels for four possible structural genes and a TF of this biosynthetic pathway in MHR4 plants may indicate that increased flavonoid content is part of the abiotic stress response syndrome. In this regard, we have observed that extracts from untreated MHR plants are consistently more pigmented than from HS plants, as was reported for MHR *A. myosuroides*.[73]

4.6 Multifunctional proteins

Five protein spots were identified as multifunctional proteins (Table 4), or those with a traditional enzymatic activity as well as additional role(s) in seemingly unrelated processes like transcriptional regulation.[125] Fructose 1,6-bisphosphate aldolase (FBPA) is a well-known enzyme of glycolysis, but also functions as a TF.[126] In *A. fatua* DIGE gels, three separate spots were identified as FBPA, one of which (spot 550; 42 kDa, pI 5.2) was elevated in MHR4 plants and contained an oxidized methionine at position 62 (supporting information, file 3). Oxidized FBPA has been localized in brain tissue where it is thought to represent a biomarker for oxidative stress.[127] Protein spot 544 (42 kDa, pI 5.1) was also identified as FBPA, with the same molecular weight but a slightly lower pI than spot 550 (Fig. 2). Significantly, levels of spots 448 and 544 were lower in MHR4 than HS1 plants, and met[62] from spot 544 was reduced, which accounts for its altered pI.[128] Thus, it is tempting to speculate that higher levels of oxidized FBPA in MHR4 plants may represent a biomarker for an oxidative stress-responsive pathway.

Spots 556 and 735 were identified as members of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) multifunctional protein family, a key enzyme of glycolysis recently shown in animal systems to be involved in mRNA stability, genomic integrity and transcriptional regulation.[129] In plants, oxidative stress results in post-translational S-nitrosylation of GAPDH and rapid nuclear relocalization, where it participates in stress signal transduction or protection.[130]

4.7 Considerations for autogamous polyploid species

With one exception (*Echinochloa crus-galli*[131]), transcriptome analyses of HR to date have been conducted on diploid, allogamous species, such as *Eleusine indica* L.,[132] *L. rigidum*[22,133] and *A. myosuroides*.[23] As such, they reflect the relatively rapid evolution of HR in species with high levels of standing genetic variation, the ability to rapidly spread and create large populations and quickly fix advantageous alleles.[134] In contrast, the evolution of HR has been slower in autogamous, hexaploid *A. fatua* than in diploid weedy species.[135] Polyploidy itself may contribute to this phenomenon, since the dilution effect of susceptible homoeologous genes likely reduces absolute resistance levels in heterozygotes, so that nascent resistant individuals are less likely to survive. In simulation models, Renton *et al.*[136] noted that the rate of polygenic HR evolution can be predicted by the initial frequency and strength of R genes, degree of genetic dominance, epistasis and

similar genetic factors. Unfortunately most of these determinants are unknown, especially for weedy polyploid species. Once established, *A. fatua* resistance spread within and among fields is slower than for other species, likely due to *A. fatua*'s autogamy, limited seed production and dispersal and extended seed dormancy.[137] Nonetheless, the time periods required for the appearance of MHR *A. fatua* populations have become considerably shorter in recent years. The herbicide triallate was introduced in North America in 1966 and resistant *A. fatua* populations were documented in Montana after only 30 years of intensive use.[138] Resistance to ACCase inhibitors like diclofop-methyl and others occurred after 14 or fewer years of use, and resistance to ALS inhibitors like imazamethabenz-methyl after only 8 years.[5] By 2006, the MHR *A. fatua* populations were already resistant to pinoxaden and thiencarbazone before these products were introduced into the marketplace.[19] While there are many biological, technical and agronomic reasons why resistance to different mechanisms of action evolves at different rates,[139] the *A. fatua* MHR phenotype seems to be conferred by an accumulation of alleles that confers broad-spectrum resistance at an accelerated pace. It is unlikely that such alleles have been fixed via introgression, since outcrossing rates are estimated at only 0.05% for *A. fatua* growing in a wheat crop.[140] An alternative explanation is that the components of this constitutive abiotic stress/herbicide resistance network have been fixed through a 'step-by-step' process[141] of consecutive selection events in the same individuals over time.

4.8 Candidate NTSR genes

In addition to comparing constitutive transcriptome changes between untreated MHR4 and HS1 plants, we also sprayed HS1 plants with a labeled field dose of flucarbazone and conducted RT-qPCR assays on the 25 DEGs shown in Table 3. Overall, levels of 15 DEGs did not change, while seven increased and three decreased after herbicide treatment of HS1 plants (Table 3). The latter 10 DEGs have functions in xenobiotic catabolism, stress response and transcriptional regulation, and we suggest that their induction/repression by herbicide treatment indicates that they are good candidates for the initial steps of NTSR evolution in susceptible plants that survive treatment. Individual weeds able to induce or repress such genes faster, longer or constitutively provide an ideal background on which herbicide selection can continue to operate in succeeding generations. Gaines *et al.*[22] identified seven contigs with increased levels in HS *L. rigidum* plants after diclofop-methyl treatment, including P450s, GSTs and a GT. We suggest that MHR *A. fatua* and other species can provide rich sources for investigating the underlying physiology of both NTSR and plant responses to other abiotic stresses.

4.9 Co-segregation of transcriptome changes with flucarbazone resistance in F_3 families

Of the 25 RT-qPCR-validated DEGs shown in Table 3, differential expression of 21 co-segregated with the flucarbazone resistance phenotype in F_3 families. We are aware of only one other report of similar HR co-segregation studies, in which Gaines *et al.*[22] showed that differential levels of nine contigs including P450s, GT, GST, nitronate monooxygenase and others co-segregated with diclofop-methyl resistance in *L. rigidum* F_2 populations. To our knowledge, the other *A. fatua* co-segregating DEGs shown in Table 3 have not been associated with MHR or HR in any transcriptome analysis to date, and thus we suggest that they represent valuable leads for hypothesis testing about additional resistance mechanisms.

Clearly these F_3 families are segregating for loci unrelated to NTSR, and additional work will be required before any causal relationship can be assigned between the DEGs and proteins described here and MHR. Elevated DEGs do not necessarily reflect increased protein amounts or enzyme activities, although our proteome comparisons more closely represent the actual cellular environment. Using a candidate gene approach, individual features can be pursued biochemically, and the rudimentary mapping populations we have developed (Burns *et al.*, submitted) could form the basis of additional genetic or QTL-based strategies. Further, these studies do not provide direct insight into the specific mechanism(s) of flucarbazone resistance in MHR4 *A. fatua*, which has not been elucidated in any weedy species. Faster inferred rates of metabolism were observed in two naturally tolerant turfgrass species,[50] and in flucarbazone-resistant *A. fatua* populations, Nandula and Messersmith[142] speculated that resistance was due to increased metabolism based on ALS sensitivity to the herbicide. Individual metabolites of flucarbazone have not been identified from weedy species, but the primary metabolite in wheat is 2-(trifluoromethoxy)benzenesulfonamide, an enzymatic conversion likely performed by an amidohydrolase.

5 CONCLUSIONS

In general, combination transcriptome/proteome approaches can help elucidate the connections between mRNA abundance and protein accumulation, with transcription profiling providing deep coverage of primary, interacting and regulatory genes while proteomics generally identifies the more abundant elements.[143] The correlation between specific transcripts and proteins from the same experimental system is normally less than 60%,[144] and the number of identified proteins is usually smaller than for transcripts, due to technical limitations like protein solubility and spot resolution, and biological phenomena like protein abundance and post-transcriptional regulation.[145,146] Nonetheless, comparisons between our *A. fatua* RNA-Seq (Appendix S2) and proteome (Appendix S3) datasets revealed the co-identification of contigs with sequence identity to 21 of 28 UniProt proteome annotations. There was generally good functional agreement between *A. fatua* DEGs and proteins involved in xenobiotic catabolism, redox maintenance, biosynthetic processes, transcriptional regulation and plant response to stress. Although the co-segregation of differentially expressed proteins with MHR was not tested here, the overlap in protein and DEG functional categories shows that both groups reflect similar constitutive changes in MHR plants.

Proteome surveys revealed a number of differentially abundant proteins that were not reflected in transcriptome results, such as biosynthetic enzymes and multifunctional proteins. Together, we believe that the synergistic transcriptome/proteome combination provides a more complete representation of the MHR phenotype than either technique alone. Our results also support the idea that MHR4 plants possess an altered, constitutively regulated system of stress-related gene expression. It thus appears that strong selection has resulted in populations that are primed to respond to a number of stresses including herbicides, a conclusion that raises several interesting questions about their provenance. For example, did these plants adapt and modify preexisting stress pathways[147] involving Hsps, anthocyanin biosynthesis and similar protective responses? Or did sublethal herbicide exposure induce their *de novo* evolution? Do the significant numbers of differential TFs, multifunctional proteins and analogous factors found in MHR4 plants signify a transcriptional reprogramming of stress pathway

regulation? Answers to these and related questions await further work such as examining known abiotic stress-specific TFs[9] and their *cis*-elements in *A. fatua*.

The suites of DEGs and proteins identified here are associated with MHR, but their individual and collective contributions to the MHR phenotype, if any, remain to be determined. The current studies also do not inform the question of whether constitutive changes alone are sufficient to confer MHR or if herbicide-induced gene expression is also required.[21] The RT-qPCR-validated changes occurring in flucarbazone-treated HS1 plants may help address this distinction, while co-segregation analyses also provide candidates for further investigation. Finally, MHR4 *A. fatua* plants are also resistant to at least nine other herbicides from five different mode of action families in addition to flucarbazone. Determining the specific, and likely overlapping, genes/enzymes responsible for resistance to each of these herbicides is the subject of ongoing work in our laboratories.

ACKNOWLEDGEMENTS

The excellent technical assistance of Katie Steward, Hayden Bateman and Mohammed Refai is much appreciated. This work was partially supported by USDA-NIFA-AFRI grants 2012-67013-19467 and 2016-67013-24888, US EPA Strategic Agricultural Initiative grant X8-97873401-0, Bayer CropScience, the Montana Wheat and Barley Committee and the Montana Agricultural Experiment Station. The proteomics, metabolomics and mass spectrometry facility at MSU receives support from the Murdock Charitable Trust and Montana INBRE under award number P20GM103474 from the National Institutes of Health (NIGMS). This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFGN00000000. The version described in this paper is the first version, GFGN01000000. We have requested a current release date for this TSA project from NCBI Data supporting the results of this article are included within the article and additional files.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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