Research Article

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Differential expression of genes associated with non-target site resistance in *Poa annua* with target site resistance to acetolactate synthase inhibitors

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Abstract

BACKGROUND: *Poa annua* is a pervasive grassy, self-pollinating, weed that has evolved resistance to 10 different herbicide modes-of-action, third most of all weed species. We investigated constitutive overexpression of genes associated with non-target site resistance (NTSR) in POAAN-R3 and the response of those genes when treated with trifloxysulfuron despite the biotype having a known target site mutation in acetolactate synthase (ALS).

RESULTS: Despite having an ALS target site mutation, POAAN-R3 still had a transcriptomic response to herbicide application that differed from a susceptible biotype. We observed differential expression of genes associated with transmembrane transport and oxidation–reduction activities, with differences being most pronounced prior to herbicide treatment.

CONCLUSIONS: In the *P. annua* biotype we studied with confirmed target site resistance to ALS inhibitors, we also observed constitutive expression of genes regulating transmembrane transport, as well as differential expression of genes associated with oxidative stress after treatment with trifloxysulfuron. This accumulation of mechanisms, in addition to the manifestation of target site resistance, could potentially increase the chance of survival when plants are challenged by different modes of action.

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

Keywords: RNA-Seq; transcriptomics; non-target site resistance; turfgrass

1 INTRODUCTION

Herbicide resistance mechanisms are often described using a binary paradigm: plants survive treatment via modifications of the herbicide target site (i.e., target site resistance; TSR), such as mutations or gene amplification, or persist via non-target site mechanisms (NTSR) that include altered herbicide absorption, translocation, or metabolism.[1] However, evidence has accumulated that multiple resistance mechanisms, both TSR and NTSR, can evolve within individual weed species. This has been exemplified most in grass species.[2] For instance a single biotype of *Lolium rigidum* evolved resistance to inhibitors of acetyl co-A carboxylase (ACCase; Group #1) via both target site mutation as well as via enhanced metabolism[3] and since then reports of stacked TSR and NTSR mechanisms have steadily increased. Han *et al.*[4] reported that 70% of resistant *L. rigidum* accessions collected in crop field surveys were resistant to ACCase inhibitors via both target site mutation and enhanced metabolism. This phenomenon is not limited to the ACCase inhibiting herbicides. Multiple resistance mechanisms have been confirmed in grasses resistant to mitotic inhibitors (Group #3)[5] as well as inhibitors of acetolactate synthase (Group #2).[6]

How do multiple resistance mechanisms evolve within a weed population? From an evolutionary perspective, selection pressure

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from herbicide treatment will select for any genes endowing mechanisms that facilitate survival to increase the number of survivors within the population.[7] For example, less than 1% of *L. rigidum* biotypes with no exposure to ACCase inhibiting herbicides survived diclofop-methyl at 375 g ha^{-1}; however, a single selection event increased resistance in progeny nearly 10-fold.[8] Resistance within progeny was confirmed to be via a NTSR mechanism that also conferred resistance to another herbicide family targeting ACCase (i.e., sethoxydim), as well as cross-resistance to ALS inhibitors chlorsulfuron and imazethapyr.[8] It is still unclear if TSR and NTSR mechanisms evolve independently in separate populations and then come together in individuals through gene flow or if they can be selected concomitantly within the same populations and/or individuals. It seems likely that outcrossing species like *L. rigidum* could stack traits by evolving them independently in different individuals and then combining them, but what about species that self-pollinate?

For almost every mode-of-action, a diversity of amino acid mutations at herbicide target sites have been identified that decrease herbicide-target binding.[9] While herbicide binding may be decreased in weeds with target site mutations, it is not completely impeded; the herbicide may still be having a subtle, inhibitory, effect on the enzyme. For example, Sammons and Gaines[10] highlighted that an array of target site mutations inhibited glyphosate (Group #9) binding with enolpyruvylshikimate-3-phosphate synthase to varying degrees but none were completely inhibitory; there was some binding of glyphosate to its target regardless of mutation. Could this have facilitated evolution of both TSR and NTSR mechanisms conferring resistance to glyphosate within the same population?[11,12] Continued use of an herbicide after selection of biotypes with target site mutations may concomitantly select for expression of NTSR mechanisms that confer multiple and cross resistance to other modes of action. Recently, a cytochrome P450 gene (*CYP810V7*) was identified in *L. rigidum* that conferred cross-resistance to five modes of action.[13]

Documented on all continents including Antarctica,[14] *Poa annua* is a pervasive grassy, self-pollinating, weed that has evolved resistance to 10 different herbicide modes-of-action, third most of all weed species.[15] A biotype of *P. annua* from Tennessee (POAAN-R3) was identified as resistant to ALS and photosystem II (Group #5) inhibiting herbicides via target site mutations.[16] The Ala-205-Phe ALS mutation in POAAN-R3 resulted in broad spectrum resistance to imidazolinone, sulfonylurea, triazolopyrimidine, sulfonylamino-carbonyl-triazolinone, and pyrimidinyl (thio) benzoate herbicides. Interestingly, POAAN-R3 was also approximately 2× more tolerant to methiozolin (Group #30), an isoxazoline herbicide with high activity versus *P. annua* when compared to an herbicide-susceptible control (GR_{50} values of 826 *versus* 423 g ha^{-1}).[17]

It is often anecdotally observed that resistance to other modes-of-action occurs swiftly after initial confirmation of herbicide resistance. This could be due to traits quickly stacking due to gene flow; however, in self-pollinating species like *P. annua*, we would not expect this to be the case.[18] We hypothesize that TSR mechanisms are not sufficient to stop selection for secondary NTSR mechanisms from occurring. It may be that during the selection process for resistance to a primary mode-of-action, whether it is TSR or NTSR, the population becomes primed for subsequent resistance evolution events. In this work we investigate the constitutive overexpression of genes associated with NTSR in POAAN-R3 and the response of those genes when treated with an ALS-inhibitor, trifloxysulfuron (despite having a known target site mutation in ALS). Differential expression of genes involved in xenobiotic detoxification could potentially prime this biotype to be less susceptible to new modes-of-action. Our primary objective was to evaluate transcriptomic responses in POAAN-R3 both before and following treatment with trifloxysulfuron. A secondary objective was to evaluate the efficacy of other modes of action for controlling POAAN-R3.

2 MATERIALS AND METHODS

2.1 Plant culture

POAAN-R3 and an herbicide susceptible biotype of *P. annua* (S; University Park, PA) were cultured inside an environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) at the University of Tennessee (Knoxville, TN). The chamber was configured to provide a constant temperature of 16 °C, 65% relative humidity, and a 16 h photoperiod. Light conditions in the chamber were maintained at an average of 572 μmol m^{-2} s^{-1}. In all experiments, seed from each biotype was germinated in a peat-based growing medium (Pro-Mix BX. Premier Tech Horticulture Ltd. Rivière-du-Loup, Québec, Canada) and irrigated to facilitate germination. No supplemental nutrition was applied after seeding and clipping was withheld.

2.2 RNA sequencing (RNA-Seq) experiment

Separate trays containing 98 cells (26.2 cm^3 each) were filled with previously described growing media. Individual POAAN-R3 or S plants with a minimum of two tillers were transplanted into each cell and allowed to acclimate to the growth chamber environment before beginning the RNA-Seq experiment, which was designed similar to Duhoux *et al.*[19] Our aim in this study was to better understand transcriptomic responses of POAAN-R3 and S *P. annua* in response to an application of the ALS-inhibiting herbicide trifloxysulfuron at label rate (27.8 g ha^{-1}). There were six time points in our time-course experiment: 0, 2, 6, 12, 24, and 48 h after treatment (HAT) with five biological replicates of each *P. annua* biotype at each time point (30 samples total). Samples for the 0 HAT time point were collected by removing aboveground tissue from both *P. annua* biotypes (POAAN-R3 and S) with sterilized scissors, and placing material from each plant in a unique sampling bag (WhirlPak. Sigma Aldrich. St. Louis, MO) filled with liquid nitrogen; five samples per biotype were harvested at 0 HAT. All samples were immediately stored at −80 °C after collection. Once 0 HAT samples were collected, remaining plants were then sprayed with trifloxysulfuron (Monument 75WG. Syngenta Professional Products. Greensboro, NC) at 27.8 g ha^{-1} inside an enclosed spray chamber (Generation III track sprayer. DeVries Manufacturing, Hollandale, MN) via an 8004 EVS nozzle at 374 L ha^{-1}. Herbicide was mixed with water carrier and included non-ionic surfactant (Activator-90. Loveland Products, Greeley, CO) at 0.25% v/v per label instructions. Treated plants were placed back inside a previously described environmental growth chamber after herbicide application with 2, 6, 12, 24 and 48 HAT tissue samples collected as previously described. All aboveground biomass was flash frozen in liquid nitrogen and stored at −80 °C until RNA was extracted using an RNeasy Plant MiniKit (Qiagen, Germantown, MD) per manufacturer instructions. Concentration of RNA extracted from each sample averaged 520 ng μL^{-1}.

Library preparation and sequencing was conducted at the University of Texas-Austin Genomic Sequencing and Analysis Support Facility (https://sites.cns.utexas.edu/cbrs/genomics). Libraries

were prepared using an Illumina (Illumina Inc. San Diego, CA) TrueSeq Stranded mRNA kit and samples were sequenced using two-lanes of Illumina HiSeq 4000 with a 2 × 150 bp paired-end run. All sequences were analyzed with FASTQC quality checker[20] and trimmed using Trimmomatic.[21]

2.3 Gene expression analysis

Sequences from POAAN-R3 and S tissues sampled at 0, 2, 6, 12, 24, and 48 HAT were aligned to the reference assembly (*P. annua* transcriptome Genbank accession GCZY01000000)[22] using TopHat[23]; results for all of the five biological replicates at each time point were sorted, and counted using HTseq count.[24] Counts were then normalized using the 'edgeR'[25] package in R (version 3.4.0).[26] Differential expression levels between the POAAN-R3 and S biotypes over the time course of our experiment were determined using voom/limma (version 3.42.2)[27] using the linear modeling approach (lmfit), empirical Bayes statistics (eBayes) and best candidate selection (topTable) with a Benjamini & Hochberg (BH) correction. Gene sequences of the reference *P. annua* transcriptome were annotated with InterProScan (version 5.36–75.0).[28] Gene ontology (GO) enrichment analysis was performed with the topGo (version 2.38.1)[27] 'weight01' algorithm.

2.4 qRT-PCR experiment

A qRT-PCR experiment was conducted to confirm results of the expression analysis performed using data from our RNA-Seq experiment. Plant culture methods and materials in this experiment were identical to those described in the RNA-Seq study. *Poa annua* biotypes (POAAN-R3 and S) were seeded into 98-cell propagation tray filled with peat moss growing medium. Seedlings were acclimated to the growth chamber environment for 5 weeks and matured to a three-tiller growth stage. There were five time points in this time-course experiment: 0, 6, 12, 24, and 48 h after treatment (HAT) with 10 biological replicates of each *P. annua* biotype at each time point. Tissue samples for the 0 HAT time point were collected as previously described, frozen in liquid N, and stored at −80 °C. Remaining plants were immediately sprayed with trifloxysulfuron at 27.8 g ha^{-1} as described above. Treated plants were returned to the environmental growth chamber after herbicide application and tissue samples collected 24 and 48 HAT as previously described.

RNA was extracted from the flash frozen tissues using Qiagen RNeasy Plant Mini Kit, following the manufacturer's protocol. The extracted RNA was quantified using a Nanodrop1000 (ThermoFisher, Waltham, MA) and 100 ng of total RNA was reverse-transcribed using Quantabio qScript cDNA SuperMix (Quantabio, Beverly, MA) following the manufacturer's protocol and diluted at a 1:20 dilution. PCR primers were then optimized using Quantabio AccuStart II PCR ToughMix (Quantabio). The 10 μL reaction for optimization consisted of 1 μL of 1:20 cDNA, 5 μL AccuStart II PCR ToughMix, and 1 μL 2.5 uM primer. The 20 μL qPCR was completed with 10 μL PerfeCTa SYBR Green Fast Mix, Lox ROX, 2 μL 2.5 μM forward primer, 2 μL 2.5 μM reverse primer, and 1 μL cDNA. The fragments were quantified using a QuantStudio6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA) in triplicates.

Quantitative PCR was performed for three genes found to be differentially expressed in the RNASeq dataset: ABC-2 (ATP-binding cassette transporter) type transporter (Genbank accession GCZY01000308), Cytochrome P450 (Genbank accession GCZY01008731) and catalase isozyme 2 (Genbank accession GCZY01006429). Designed primer sequences for these genes and amplification conditions are presented in Table 1. The reference gene was elongation factor 1α (Genbank accession GCZY01034376) and was subjected to the same conditions as the other samples. The melt curve was 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s for all primers. The relative level of expression between the resistant versus the susceptible biotype was calculated using the ΔΔCt method normalized against the elongation factor 1 as the reference gene.[29]

2.5 Alternative mode of action experiment

A whole plant experiment was conducted to determine the response of POAAN-R3 and susceptible *P. annua* to modes of action other than ALS- and PSII-inhibition. *Poa annua* biotypes POAAN-R3 and S were seeded into propagation trays filled with peat moss growing medium. After maturing for 5 weeks, plugs were removed, washed free of growth medium, and transplanted into greenhouse pots filled with Sequatchie silt loam soil (fine-loamy, siliceous, semiactive, thermic humic Hapludult). Pots were placed in a glasshouse at the University of Tennessee (Knoxville, TN; 35° 57′ N Lat), and acclimated for one-week before initiating research. Average daily maximum and minimum temperatures measured 28 and 11 °C, respectively, with average humidity of 62%. Maximum and minimum daily solar radiation inside the glasshouse averaged 2498 and 32 μmol m^{-2} s^{-1}, respectively.

Pots were treated with the following herbicides: glyphosate (Roundup Pro. Bayer Environmental Sciences. St. Louis, MO) at 1120 or 2240 g ha^{-1}; glufosinate (Finale. BASF Corporation. Research Triangle Park, NC) at 1680 or 3360 g ha^{-1}; pronamide (Kerb SC. Corteva AgroSciences. Indianapolis, IN) at 1620 or 3240 g ha^{-1}; flumioxazin (Sureguard. NuFarm. Morrisville, NC) at 420 or 840 g ha^{-1}; methiozolin at 1000 or 2000 g ha^{-1} (PoaCure. Moghu Research Center, Yuseong, Daejeon, Korea); indaziflam (Specticle Flo. Bayer Environmental Sciences. Research Triangle Park, NC) at 54.5 or 109 g ha^{-1}. These rates represented 1× and 2× label maximums for early-postemergence control of *P. annua*. Per label directions, flumioxazin included nonionic surfactant (Activator-90. Loveland Products, Loveland, CO) at 0.25% v/v. Herbicides were mixed in water and applied in enclosed spray chamber as described above. At 35 days after treatment (DAT), aboveground biomass of each experimental unit was harvested at the soil line with scissors, placed in a forced air oven at a minimum temperature of 60 °C for 7 days and weighed.

The experimental design was a randomized complete block with four replications repeated in time. An arcsine square root transformation was used to improve data normality prior to conducting analysis of variance in R (version 3.4.0).[26] Mean separation was conducted using Fisher's protected least significant difference (LSD) test at the 0.05 level via the LSD test function of the 'Agricolae' package (version 1.3–2)[30] within R. ANOVA and means separation results with transformed and non-transformed data were similar; therefore, non-transformed means are presented herein for clarity.

3 RESULTS

3.1 Gene expression and ontology analyses

Of the 55 579 genes present in the reference assembly,[22] 28 006 had sufficient supporting sequence information to evaluate expression level changes. In total, 4447 genes (Table S1) were differentially expressed for at least one of the six different time points (adj. *P*-value <0.05). The number of genes that were either up- or down-regulated is described in Fig. 1. There were more

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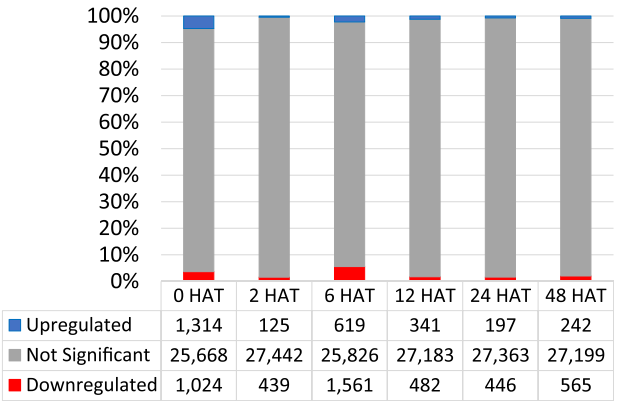
Table 1. Genes selected for quantitative RT-PCR confirmation of differential gene expression among herbicide-resistant (POAAN-R3) and susceptible biotypes of *Poa annua*

Gene	Genbank accession	Forward/ Reverse	Primer sequence (5′–>3′)	Amplification conditions
Elongation factor 1α	GCZY01034376	F	GTTGCAACAAGATGGATGCC	95 °C, (95 °C 10 s, 54 °C for 20 s, 72 °C 10 min) × 45
		R	GCCCTCAAAGCCAGAGATT	
ABC-2 type transporter	GCZY01000308	F	GAAGACGATGACGTAGGTGAAG	95 °C, (95 °C 10 s, 64 °C for 20 s, 72 °C 10 min) × 45
		R	CCTCATCTTCAGACCGTTGATAC	
		R	CGGGTAGTATGCGAGCTAAAT	
Catalase isozyme 2	GCZY01006429	F	CCGTCGTAGTGGTTGTTCTT	95 °C, (95 °C 10 s, 58 °C for 20 s, 72 °C 10 min) × 40
		R	GCTCTTCGTGCAGGTGAT	
Cytochrome P450	GCZY01008731	F	CAGGTTCCATATCACCCATTCC	95 °C, (95 °C 10 s, 60 °C for 20 s, 72 °C 10 min) × 45
		R	AGCTCTGAACCACTACGTCT	

Genbank accession and primers used for amplification are presented for each gene.



	0 HAT	2 HAT	6 HAT	12 HAT	24 HAT	48 HAT
Upregulated	1,314	125	619	341	197	242
Not Significant	25,668	27,442	25,826	27,183	27,363	27,199
Downregulated	1,024	439	1,561	482	446	565

Figure 1. The number of differentially expressed genes in herbicide-resistant (POAAN-R3) and susceptible biotypes of *Poa annua*. Expression analysis was performed on a total of 28 006 genes.

genes differentially expressed at 0 HAT (2338 genes) and 6 HAT (2180 genes) than at any other time points during the experiment (564, 823, 643 and 807 for 2, 12, 24 and 48 HAT, respectively). Except for the non-treated plants (0 HAT), there were more genes that were down-regulated in the POAAN-R3 biotype (or up-regulated in the susceptible biotype) when treated with the herbicide for all time points. Before treatment (0 HAT), there were more genes expressed at a higher level in the POAAN-R3 biotype (1314) than the susceptible control (1024).

Gene ontology (GO) enrichment analysis identified several biological processes were constitutively augmented in POAAN-R3 compared to the S control at 0 HAT (Fig. 2). The most important category (in terms of the number annotations) was genes involved in diverse activities related to oxidation–reduction processes with a total of 1685 annotations identified. General transport was well represented in the GO enrichment analysis with a total of 1423 annotations: 1185 for transmembrane transport, 213 for metal ion transport, 22 for sulfate transport and three for nucleoside transmembrane transport. Carbohydrate metabolic process appeared to be reduced with 803 annotations. The analysis of GO cellular components indicate that overexpressed genes in POAAN-R3 clearly describe activities that are membrane bound. For the overexpressed gene set, all annotations (2533) were associated with being membrane bound, whereas activities occurring in the cytoplasm appear to be reduced in POAAN-R3 as compared to the susceptible line. Lastly, the molecular analysis agrees with the other two ontologies revealing oxidoreductase activities (1741 annotations) and transmembrane transporter activities (1064) being most important.

The response of POAAN-R3 over time is also different than the susceptible line. From 2 to 48 HAT, differential expression analysis and GO enrichment indicated an elevation in the level of activity for processes related to oxidation–reduction but also cell redox homeostasis and response to oxidative stresses (Fig. 3). Genes involved in cellulose biosynthesis and cell wall modification are comparatively enriched in the resistant biotype, POAAN-R3 as well. Finally, genes related to defense against fungi and bacteria were also overexpressed at 2, 24 and 48 HAT.

3.2 qRT-PCR experiment

Three genes were selected to validate expression differences between POAAN-R3 and the susceptible biotype. For this purpose, the entire experiment was repeated including the herbicide application on young plantlets. Two of the selected genes were potentially involved in non-target site resistance mechanisms, an ABC-2 type transporter and a cytochrome P450 monooxygenase,[32] whereas the third, a catalase, converts peroxide (a strong oxidizer) to water and O_2.[33] These three genes were identified as being differentially expressed in our RNA-Seq analysis and enriched in our GO analysis. In the qRT-PCR experiment, the ABC-2 type transporter was significantly over-expressed at 0 and 24 HAT, with values of 3.2 and 5.6 times the values obtained with the susceptible biotype. At the same time points, RNASeq analysis had revealed 8.6 and 1.8 fold over-expression of this ABC-2 type transporter (Fig. 4). For the catalase-coding gene, qRT-PCR revealed significant over-expression (4.6 fold) at 48 HAT whereas RNA-Seq had indicated a 3.5 fold change. For the cytochrome P450, no significant change in expression was detected in the qRT-PCR experiment while fold changes of 4.2 and 5.2 were obtained after RNAseq data analysis, at 24 and 48 HAT, respectively.

3.3 Alternative mode of action experiment

Aboveground biomass varied among experimental factors with main effects of application rate, *P. annua* biotype, and herbicide significant at $\alpha = 0.05$ (Table 2). Glyphosate, glufosinate, and flumioxazin reduced biomass of both POAAN-R3 and the herbicide-susceptible biotype 72% to 100% (≤ 0.07 g compared

GO.ID	Biological Process Terms	Annotated	Significant	Expected	classic	Expression
GO:0055114	oxidation-reduction process	1685	41	27.27	0.0042	Up
GO:0055085	transmembrane transport	1185	30	19.18	0.0121	Up
GO:0030001	metal ion transport	213	11	3.45	0.001	Up
GO:0008272	sulfate transport	22	2	0.36	0.0487	Up
GO:0009439	cyanate metabolic process	4	2	0.06	0.0015	Up
GO:0008283	cell proliferation	3	1	0.05	0.0478	Up
GO:1901642	nucleoside transmembrane transport	3	1	0.05	0.0478	Up
GO:0050832	defense response to fungus	2	1	0.03	0.0321	Up
GO:0090266	regulation of mitotic cell cycle spindle...	2	1	0.03	0.0321	Up
GO:0042742	defense response to bacterium	2	1	0.03	0.0321	Up
GO:0006012	galactose metabolic process	2	1	0.02	0.022	Down
GO:0006438	valyl-tRNA aminoacylation	2	1	0.02	0.022	Down
GO:0042549	photosystem II stabilization	2	1	0.02	0.022	Down
GO:0043486	histone exchange	2	1	0.02	0.022	Down
GO:0009264	deoxyribonucleotide catabolic process	2	1	0.02	0.022	Down
GO:0006450	regulation of translational fidelity	2	1	0.02	0.022	Down
GO:0006433	prolyl-tRNA aminoacylation	4	1	0.04	0.043	Down
GO:0005975	carbohydrate metabolic process	803	18	8.78	0.023	Down

GO.ID	Cellular Component Terms	Annotated	Significant	Expected	classic	Expression
GO:0016020	membrane	2533	51	34.14	1.80E-07	Up
GO:0005786	signal recognition particle, endoplasmic...	6	1	0.05	0.0478	Down
GO:0009654	photosystem II oxygen evolving complex	43	2	0.35	0.0471	Down
GO:0000786	nucleosome	64	4	0.52	0.0016	Down
GO:0005737	cytoplasm	1111	11	9.04	0.0492	Down

GO.ID	Molecular Funciton Terms	Annotated	Significant	Expected	classic	Expression
GO:0016491	oxidoreductase activity	1741	45	26.26	0.02916	Up
GO:0022857	transmembrane transporter activity	1064	31	16.05	0.00925	Up
GO:0016758	transferase activity, transferring hexos...	526	18	7.93	0.00061	Up
GO:0003700	DNA-binding transcription factor activit...	409	14	6.17	0.00383	Up
GO:0020037	heme binding	518	14	7.81	0.02602	Up
GO:0005506	iron ion binding	426	12	6.43	0.02875	Up
GO:0016887	ATPase activity	312	11	4.71	0.00191	Up
GO:0016705	oxidoreductase activity, acting on paire...	394	11	5.94	0.03734	Up
GO:0043565	sequence-specific DNA binding	225	9	3.39	0.0073	Up
GO:0005507	copper ion binding	45	4	0.68	0.00464	Up
GO:0010181	FMN binding	38	3	0.57	0.01941	Up
GO:0003779	actin binding	52	3	0.78	0.04364	Up
GO:0004471	malate dehydrogenase (decarboxylating) (...	12	2	0.18	0.01354	Up
GO:0004144	diacylglycerol O-acyltransferase activit...	16	2	0.24	0.02366	Up
GO:0004525	ribonuclease III activity	19	2	0.29	0.03274	Up
GO:0015116	sulfate transmembrane transporter activi...	22	2	0.33	0.04294	Up
GO:0008430	selenium binding	2	1	0.03	0.02994	Up
GO:0004555	alpha,alpha-trehalase activity	2	1	0.03	0.02994	Up
GO:0005337	nucleoside transmembrane transporter act...	3	1	0.05	0.04457	Up
GO:0008083	growth factor activity	3	1	0.05	0.04457	Up
GO:0010242	oxygen evolving activity	2	1	0.02	0.02233	Down
GO:0070402	NADPH binding	2	1	0.02	0.02233	Down
GO:0004832	valine-tRNA ligase activity	2	1	0.02	0.02233	Down
GO:0008108	UDP-glucose:hexose-1-phosphate uridylylt...	2	1	0.02	0.02233	Down
GO:0050080	malonyl-CoA decarboxylase activity	2	1	0.02	0.02233	Down
GO:0004853	uroporphyrinogen decarboxylase activity	4	1	0.04	0.04417	Down
GO:0008253	5'-nucleotidase activity	4	1	0.04	0.04417	Down
GO:0004827	proline-tRNA ligase activity	4	1	0.04	0.04417	Down
GO:0005542	folic acid binding	4	1	0.04	0.04417	Down
GO:0004471	malate dehydrogenase (decarboxylating) (...	12	2	0.13	0.00769	Down
GO:0071949	FAD binding	25	2	0.28	0.03178	Down
GO:0008641	ubiquitin-like modifier activating enzym...	26	2	0.29	0.03418	Down
GO:0004743	pyruvate kinase activity	20	3	0.22	0.00138	Down
GO:0030955	potassium ion binding	20	3	0.22	0.00138	Down
GO:0010333	terpene synthase activity	34	3	0.38	0.00646	Down
GO:0003755	peptidyl-prolyl cis-trans isomerase acti...	101	4	1.13	0.02711	Down
GO:0000287	magnesium ion binding	94	6	1.06	0.00067	Down
GO:0008234	cysteine-type peptidase activity	205	6	2.3	0.03211	Down
GO:0004553	hydrolase activity, hydrolyzing O-glycos...	444	11	4.99	0.03621	Down

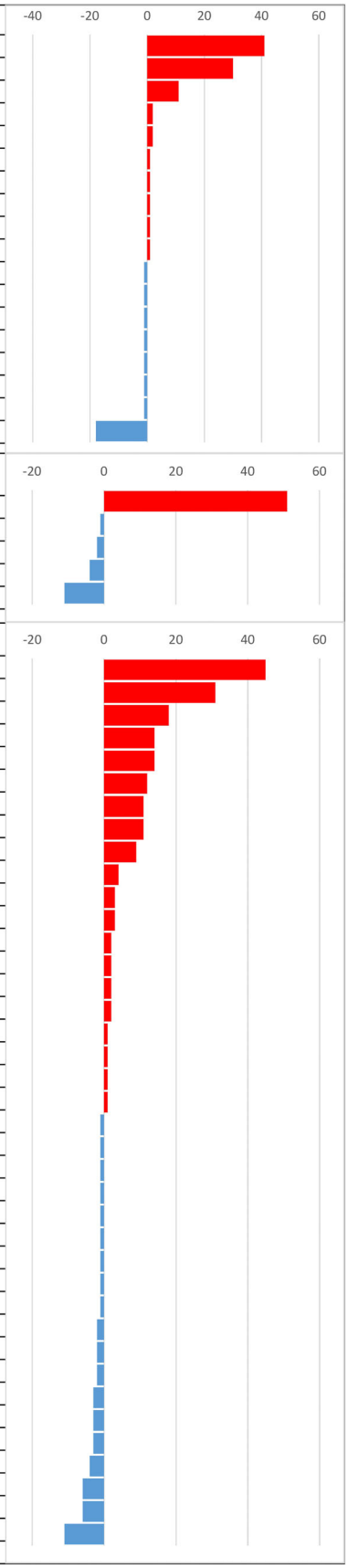


Figure 2. Biological processes, cellular component and molecular function gene ontology enrichment of genes differentially expressed between herbicide-resistant and -susceptible *Poa annua* biotypes before treatment calculated with topGO[31] sorted by the number of significant terms for each category. Positive and values indicate values indicate GO categories overexpressed and suppressed in the resistant (POAAN-R3) biotype, respectively, compared to the susceptible biotype.

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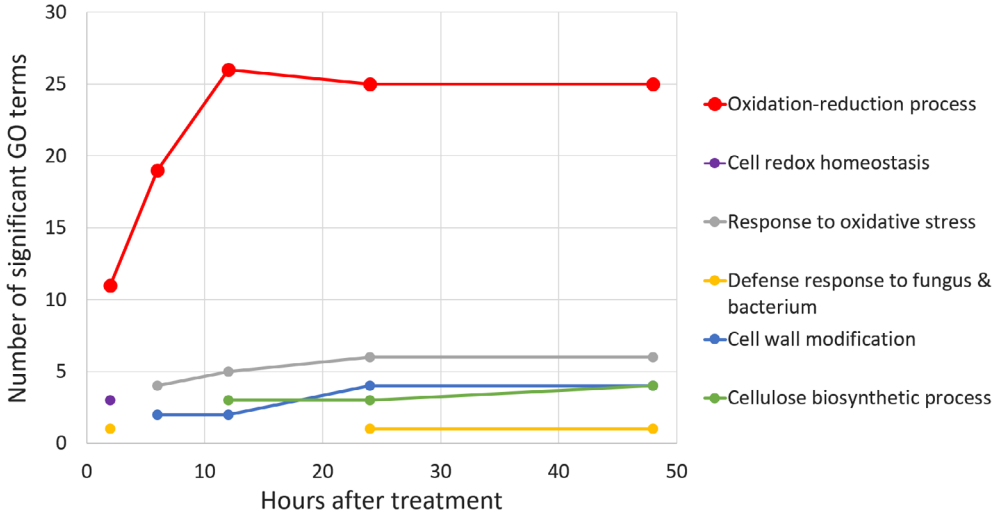

Figure 3. Number of significant GO terms enriched in genes overexpressed in the resistant biotype POAAN-R3 compared to the susceptible biotype at 2, 6, 12, 24 and 48 h after treatment, as calculated with topGO.[31]

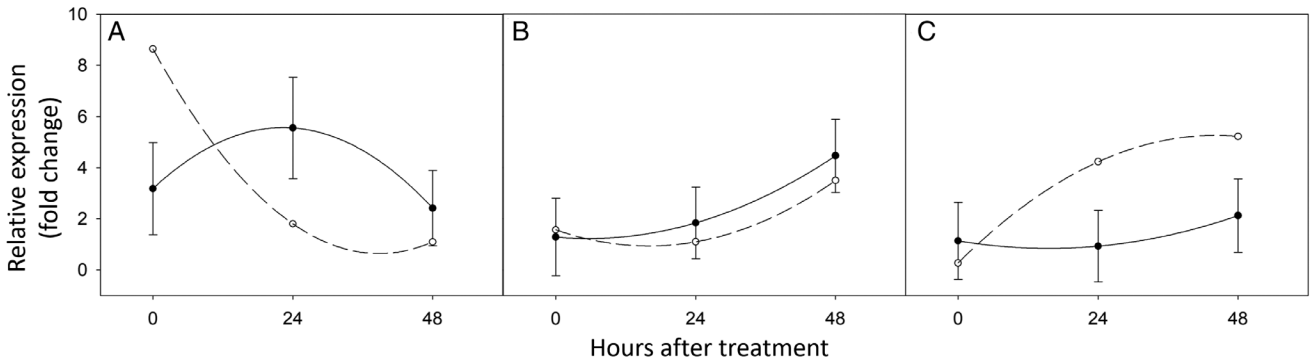

Figure 4. Relative expression between herbicide-resistant (POAAN-R3) and -susceptible *Poa annua* biotypes for three selected genes. Gene expression level was measured by qRT-PCR (closed dots, solid line), normalized against elongation factor 1α and ratios calculated using the $\Delta\Delta$Ct method[29]; relative expression changes was also calculated from RNASeq data (open dots, dash line). Panel A: ABC-2 type transporter (Genbank accession GCZY01000308), Panel B: catalase isozyme 2 (Genbank accession GCZY01006429) and Panel C: Cytochrome P450 (Genbank accession GCZY01008731).

to ≥ 0.25 g for non-treated controls). Biomass values were 2× greater on POAAN-R3 than herbicide-susceptible *P. annua* following treatment with pronamide at 1620 g ha^{-1}; however, this difference was negated with an increase in application rate to 3240 g ha^{-1}. Biomass values were also 60% greater on POAAN-R3 than the susceptible biotype following treatment with indaziflam at 54 g ha^{-1} and increasing application rate to 108 g ha^{-1} only accentuated the difference.

4 DISCUSSION

Plants have evolved complex mechanisms to maintain homeostasis when under different external abiotic or biotic stresses.[34] The transcriptional response to the pressure exerted on the plant in these conditions can include changes in the expression of many genes.[34] This phenomenon could be exemplified by the response of weeds after application of herbicide, many of which will induce dramatic changes at the cellular level before the plant either dies or adapts to survive. The production of reactive oxygen species (ROS) has been described following the application of several groups of herbicides[35] and, concomitant to previously described resistance mechanisms (reviewed in Powles and Yu[9] and Delye *et al.*[32]), the expression of genes involved in ROS metabolism would be required to maintain homeostasis. Therefore, it is possible that resistant plants with target site mutations may also be able to tolerate, by the means of specific gene expression, collateral effects of xenobiotics, rendering them more tolerant to other stresses that could come from herbicide active ingredients of different structures.

Interestingly, our data showed that there were more genes differentially expressed in a resistant *P. annua* biotype at 0 HAT (2338) versus any other time point during the experiment. Our first time point was the only one where the number of up-regulated genes was higher than the number of down-regulated genes in our resistant line (relative to susceptible). Although the susceptible and resistant biotypes were collected at different locations, this observation suggests that the susceptible biotype reacted more strongly to the herbicide challenge.

However, POAAN-R3 still had a transcriptomic response to herbicide application; furthermore, the response was different then the susceptible line. We hypothesize that even though the amount of trifloxysulfuron was not sufficient to kill POAAN-R3 due to the presence of the Ala-205-Phe ALS mutation, plants are overexpressing detoxification pathways to clear trifloxysulfuron

Table 2. Differences in aboveground biomass of herbicide-susceptible (S) and –resistant (POAAN-R3) *Poa annua* following treatment with modes of action other than acetolactate synthase or photosystem II inhibition in a glasshouse located in Knoxville, TN

			Biomass (g)[¶]	
Herbicide[†]	WSSA group[‡]	Rate (g ha^{-1})[§]	Susceptible	POAAN-R3
Flumioxazin	14	420	0.00	0.07
		840	0.00	0.02
Glufosinate	10	1680	0.00	0.01
		3360	0.00	0.00
Glyphosate	9	1400	0.00	0.00
		2800	0.01	0.04
Indaziflam	29	54	0.10	0.16
		108	0.05	0.21
Methiozolin	30	1000	0.16	0.16
		2000	0.03	0.07
Pronamide	3	1620	0.07	0.14
		3240	0.04	0.03
Non-treated	–	–	0.25	0.27
	Main effect of herbicide		***	
	Main effect of rate		*	
	Main effect of biotype		**	

[†] Herbicides applied to tillering annual bluegrass plants inside an enclosed spray chamber at 374 L ha^{-1}.
[‡] Mode of action group as classed by the Weed Science Society of America.
[§] Rates represent 1× and 2× label maximums for annual bluegrass control in turfgrass.
[¶] Aboveground biomass of each experimental unit was harvested at the soil line with scissors, placed in a forced air oven at a minimum temperature of 60 °C for 7 days and weighed.

from the cell and mitigate any inhibition it may be still performing. If this secondary detoxification is beneficial, this opens up the possibility for natural selection of individuals with a strong secondary resistance response, potentially priming them for cross resistance to novel modes-of-action.

Gene ontology analyses for biological processes indicated that several metabolic activities were significantly different between POAAN-R3 and the susceptible biotype (Figs 2 and 3). The most important GO category that differed was related to oxidation–reduction processes at 0 HAT and several time points thereafter. As mentioned before, many herbicides induce production of reactive oxygen species (ROS), including glyphosate, glufosinate, photosystem II and ALS inhibitors.[35] The biotype POAAN-R3 had been previously described as having evolved resistance to photosystem II and ALS inhibitors[16] and was effectively controlled by glyphosate and glufosinate in our research (Table 2). This response suggests that protection against oxidative stress, alone, does not overcome the challenge imposed by glyphosate or glufosinate.

The second most important biological process was related to transmembrane transport, an observation that was supported by the enrichment of the GO terms membrane, integral component of membrane, and anchored component of membrane in the cellular component aspect of the ontology analysis (Fig. 3). Transporters, ATP-binding cassette transporters in particular, have been associated with herbicide resistance via translocation of active ingredients to less metabolically active regions such as the vacuole or the cell wall.[9,36] In *Arabidopsis thaliana*, a gain-of-function mutation in an ABC-transporter conferred auxinic herbicide resistance.[37] Additionally, rapid accumulation of glyphosate in the vacuole is suspected through a similar mechanism to confer resistance in *Conyza canadensis*.[38]

The entire experiment was repeated and expression levels of three genes suspected to be involved in herbicide response were measured using quantitative PCR. Results obtained for the catalase gene (Fig. 4, Panel B) were highly consistent between qRT-PCR and RNASeq analysis. For the selected cytochrome P450 (Fig. 4, Panel C), 4.2 to 5.2 fold overexpression after 24 and 48 h was detected using RNASeq while lower values (up to 2.1 fold overexpression) were revealed using qRT-PCR. For the ABC-transporter gene selected (Fig. 4, Panel A), RNAseq indicated a higher constitutive expression than qRT-PCR (8.6 fold versus 3.2), whereas the latter method showed higher overexpression at the 24 h time point (1.8 for RNASeq versus 5.6 for qRT-PCR). A possible explanation of the difference may reside in the specificity of the assay, which may be hindered by the fact that the targeted genes are part of large families, especially cytochrome P450s and ABC-transporters. In all cases, however, the selected genes were expressed at higher levels in the resistant biotype.

5 CONCLUSIONS

Overall, this dataset provides additional evidence of a weed species accumulating multiple mechanisms to overcome the stress of herbicide application. We show that selection of NTSR mechanisms may still be possible even in the presence of a target-site mutation, and especially worrying conclusion due to the possibility of NTSR mechanisms conferring cross resistance. In the *P. annua* biotype we studied with confirmed target site resistance to ALS inhibitors, we also observed constitutive expression of genes regulating transmembrane transport, as well as differential expression of genes associated with oxidative stress after treatment with trifloxysulfuron. This accumulation of mechanisms

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could potentially increase the chance of *P. annua* survival when the plants are challenged by different modes of action.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

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

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