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.

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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 metabolism3 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
from herbicide treatment will select for any genes ending mechanisms that facilitate survival to increase the number of survivors within the population. For example, less than 1% of L. rigidum biotypes with no exposure to ACCCase inhibiting herbicides survived diclofop-methyl at 375 g ha$^{-1}$; however, a single selection event increased resistance in progeny nearly 10-fold. Resistance within progeny was confirmed to be via a NTSR mechanism that also conferred resistance to another herbicide family targeting ACCCase (i.e., sethoxydim), as well as cross-resistance to ALS inhibitors chlorsulfuron and imazethapyr. 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. 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 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? 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.

Documented on all continents including Antarctica, 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. A biotype of P. annua from Tennessee (POAAN-R3) was identified as resistant to ALS and photo-system II (Group #5) inhibiting herbicides via target site mutations. The Ala-205-Phe ALS mutation in POAAN-R3 resulted in broad spectrum resistance to imidazolinone, sulfonylurea, triazolopyrimidine, sulfonlamino-carbonyl-triazolinone, and pyrimidinyl (thio) benzoate herbicides. Interestingly, POAAN-R3 was also approximately 2x 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$^{-1}$).

It is often anecdotaly 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. 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$^{2}$ 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 aclimate to the growth chamber environment before beginning the RNA-Seq experiment, which was designed similar to Duhoux et al.. 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 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.

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) True-Seq Stranded mRNA kit and samples were sequenced using two-lanes of Illumina HiSeq 4000 with a 2 x 150 bp paired-end run. All sequences were analyzed with FASTQC quality checker and trimmed using Trimmomatic.29

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)26 using TopHat25; 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 edger22 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 (limfit), 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 μM 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 QuantStudio3 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 1α 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; praminodine (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; methiolzin 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 1x and 2x 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)20 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
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,\(^3\) whereas the third, a catalase, converts peroxide (a strong oxidizer) to water and \(\text{O}_2\).\(^2\) 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 indicated 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.

### 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-resistant biotype 72% to 100% (\(\leq 0.07\) g compared to water and \(\text{O}_2\).
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 topGO31 sorted by the number of significant terms for each category. Positive and values indicate GO categories overexpressed and suppressed in the resistant (POAAN-R3) biotype, respectively, compared to the susceptible biotype.

**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 topGO31 sorted by the number of significant terms for each category. Positive and values indicate GO categories overexpressed and suppressed in the resistant (POAAN-R3) biotype, respectively, compared to the susceptible biotype.
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. The transcriptional response to the pressure exerted on the plant in these conditions can include changes in the expression of many genes. 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 and, concomitantly to previously described resistance mechanisms (reviewed in Powles and Yu and Delye et al.), 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 than the susceptible line. We hypothesize that even though the amount of trifloxsulfuron 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 trifloxsulfuron.
The biotype POAAN-R3 had been associated with herbicide resistance via translocation of active ingredients to less metabolically active regions such as the vacuole or the cell wall. Additionally, rapid accumulation of glyphosate in the vacuole is suspected through a similar mechanism to that described for the vacuole or cell wall.9,36

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 previously, many herbicides induce production of reactive oxygen species (ROS), including glyphosate, glufosinate, and ALS inhibitors.35 The biotype POAAN-R3 had been previously described as having evolved resistance to photosystem II and ALS inhibitors.16 The biotype POAAN-R3 had been previously described as having evolved resistance to photosystem II and ALS inhibitors 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 showed higher 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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**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.

| 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 |

† 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 1x and 2x label maximums for annual bluegrass control in turfgrass.

Main effect of herbicide ***
Main effect of rate +
Main effect of biotype **

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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.

**REFERENCES**

1. Heap I, Global perspective of herbicide-resistant weeds. *Pest Manag Sci* **70**(10): 1306–1315 (2014).
2. Yu Q and Powles S, Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. *Plant Physiol* **166**(1106–1118) (2014).
3. Tardif FJ and Powles SB, Herbicide multiple-resistance in a *Lolium rigidum* biotype is endowed by multiple mechanisms: isolation of a sub-set with resistant acetol-CoA carboxylase. *Physiol Plant* **91**(488–494) (1994).
4. Han H, Yu Q, Owen MJ, Cawthray GR and Powles SB, Widespread occurrence of both metabolic and target-site herbicide resistance mechanisms in *Lolium rigidum* populations. *Pest Manag Sci* **72**(255–263) (2016).
5. Chen J, Chu Z, Han H, Goggin DE, Yu Q, Sayer C et al., A Val-202-Phe alpha-tubulin mutation and enhanced metabolism confer dinitroaniline resistance in a single *Lolium rigidum* population. *Pest Manag Sci* **67**(645–652) (2019).
6. Christopher JT, Powles SB and Holtum JAM, Resistance to acetolactate synthase-inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. *Plant Physiol* **100**(1909–1913) (1992).
7. Powles SB and Matthews JM, Multiple herbicide resistance in annual ryegrass (*Lolium rigidum*): a driving force for the adoption of integrated weed management strategies, in *Resistance 91: Achievements and Developments in Combating Pesticide Resistance*, ed. by Denholm I, Devonshire A and Holloman D. Elsevier Press, London, pp. 75–87 (1992).
8. Neve P and Powles S, High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance. *Heredity* **95**(485–492) (2005).
9. Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Ann Rev Plant Biol* **61**(317–347) (2010).
10. Sammons RD and Gaines TA, Glycophosphate resistance: state of knowledge. *Pest Manag Sci* **70**(1367–1377) (2014).
11. McElroy JS and Hall ND, *Echinocloa colon* with reported resistance to glyphosate conferred by aldol-keto reductase also contains a Pro-106-Thr ESPS target site mutation. *Plant Physiol* **182**(431–436) (2015).
12. Pan L, Yu Y, Han H, Mao L, Nyporko A, Fan LJ et al., Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinocloa colon*.* Plant Physiol* **181**(1519–1524) (2019).
13. Han H, Yu Q, Beffa R, Gonzalez S, Maiwald F, Wang J et al., Cytochrome P450 CYP81A10v7 in *Lolium rigidum* confers metabolic resistance to herbicides across at least five modes of action. *Plant J* **105**(79–92) (2021).
14. Molina-Montenegro MA, Carrasco-Urra F, Acuña-Rodríguez I, Oses R, Torres-Diaz C and Chwedorzewska KJ, Assessing the importance of human activities for the establishment of the invasive *Poa annua* in Antarctica. *Polar Res* **33**(1–7) (2014).
15. Heap I, International Survey of Herbicide Resistant Weeds. Available: www.weedsscience.org (23 February 2021).
16. Brosnan JT, Vargas JJ, Breeden GK, Grier L, Aponte RA, Tresch S et al., A new amino acid substitution (Ala-205-Phe) in acetolactate synthase (ALS) confers broad spectrum resistance to ALS-inhibiting herbicides. *Planta* **243**(1499–1509) (2016).
17. Brosnan JT, Vargas JJ, Breeden GK, Boggs SL, Staton MA, Wadi PA et al., Controlling herbicide-resistant annual bluegrasses (*Poa annua*) phenotypes with methiozolin. *Weed Technol* **31**(470–476) (2017).
18. Delye C, Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. *Pest Manag Sci* **69**(176–187) (2013).
19. Duhoz A, Carrere S, Gouzy J, Bonin L and Delye C, RNA-Seq analysis of ryegrass transcriptomic response to an herbicide inhibiting acetolactate-synthase identifies transcripts linked to non-target-site-based resistance. *Plant Mol Biol* **87**(473–487) (2015).
20. Andrews S, FASTQC: A quality control tool for high throughput sequence data (2010). Available: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.
21. Bolger AM, Lahse M and Usadel B, Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**(2114–2120) (2014).
22. Chen S, McElroy JS, Dane F and Goertzen LR, Transcriptome assembly with its two diploid progenitor species, *Poa supina* Schrad and *Poa infirma* Kunth. *Plant Genome* **9**(1) (2016). https://doi.org/10.3835/plantgenome.2015.06.0050.
23. Kim D, Perteta G, Trapnell C, Pimentel H, Kelley R and Salzberg SL, TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* **14**(R36) (2013).
24. Anders S, Pyl PT and Huber W, HTSeq: a python framework to work with high-throughput sequencing data. *Bioinformatics* **31**(166–169) (2015).
25. McCarthy DJ, Chen Y and Smyth GK, Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* **40**(4288–4297) (2012).
26. R Core Team, R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna (2020).
27. Law CW, Chen Y, Shi Y and Smyth GK, Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol **15**(1029) (2014).
28. Mitchell AL, Attwood TK, Babbitt PC, Blum M, Bork P, Bridge A et al., InterPro in 2019: improving coverage, classification and access to protein sequence annotations. *Nucleic Acids Res* **47**(D351–D360) (2019).
29. Livak KJ and Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**(402–408) (2001).
30. de Mendiburu F and Muhammed M, agricolae: statistical procedures for agricultural research (2020).
31. Alexa A and Rahnenfuhrer J, topGO: enrichment analysis for gene ontology: R package (2020).
32. Delye C, Jasieniuk M and Le Corre V, Deciphering the evolution of herbicide resistance in weeds. *Trends Genet* **29**(649–658) (2013).
33. Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Van Breusegem F and Noter D, Controlling herbicide-resistant annual bluegrass, *Poa annua*, and its two diploid progenitor species, *Poa supina* Schrad and *Poa infirma* Kunth. *Plant Genome* **9**(1) (2016). https://doi.org/10.3835/plantgenome.2015.06.0050.
34. Mitchelle AL, Attwood TK, Babbitt PC, Blum M, Bork P, Bridge A et al., InterPro in 2019: improving coverage, classification and access to protein sequence annotations. *Nucleic Acids Res* **47**(D351–D360) (2019).
35. Livak KJ and Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**(402–408) (2001).
36. de Mendiburu F and Muhammed M, agricolae: statistical procedures for agricultural research (2020).
37. Alexa A and Rahnenfuhrer J, topGO: enrichment analysis for gene ontology: R package (2020).
38. Delye C, Jasieniuk M and Le Corre V, Deciphering the evolution of herbicide resistance in weeds. *Trends Genet* **29**(649–658) (2013).
39. Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Van Breusegem F and Noter D, Controlling herbicide-resistant annual bluegrass, *Poa annua*, and its two diploid progenitor species, *Poa supina* Schrad and *Poa infirma* Kunth. *Plant Genome* **9**(1) (2016). https://doi.org/10.3835/plantgenome.2015.06.0050.
40. Jovi B, Soós V, Szegő D, Pálfi E, Szigiti E, Rácz I et al., Role of transporters in paraquat resistance of horseweed *Conyza canadensis* (L.) Cronq. *Pestic Biochem Physiol* **88**(57–65) (2007).
41. Ito H and Gray WM, A gain-of-function mutation in the Arabidopsis pleiotropic drug resistance transporter PDR9 confers resistance to aminic herbicides. *Plant Physiol* **142**(63–74) (2006).
42. Ge X, d’Avignon DA, Ackerman JJ and Sammons RD, Rapid vascular sequestration: the horseweed glyphosate resistance mechanism. *Pest Manag Sci* **66**(345–348) (2010).