MULTIPLE HERBICIDE-RESISTANT WEEDS AND NON-TARGET SITE RESISTANCE MECHANISMS: A GLOBAL CHALLENGE FOR FOOD PRODUCTION

EDITED BY: Joel Torra, Maria D. Osuna, Aldo Merotto Junior and Martin Vila-Aiub

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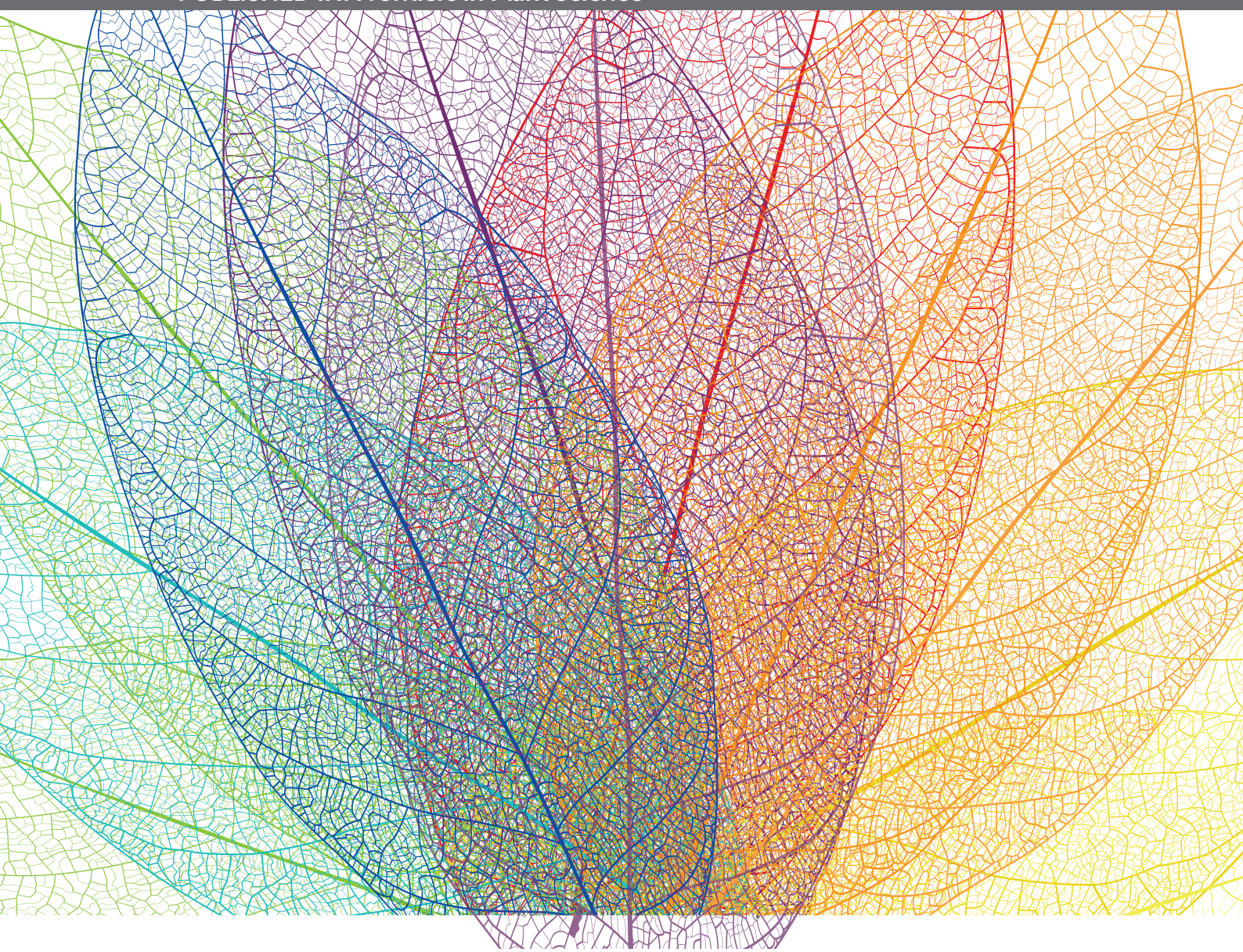

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MULTIPLE HERBICIDE-RESISTANT WEEDS AND NON-TARGET SITE RESISTANCE MECHANISMS: A GLOBAL CHALLENGE FOR FOOD PRODUCTION

Topic Editors:
Joel Torra, Universitat de Lleida, Spain
Maria D. Osuna, Center for Scientific and Technological Research of Extremadura (CICYTEX), Spain
Aldo Merotto Junior, Federal University of Rio Grande do Sul, Brazil
Martin Vila-Aiub, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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Editorial: Multiple Herbicide-Resistant Weeds and Non-target Site Resistance Mechanisms: A Global Challenge for Food Production

Joel Torra[1], *María Dolores Osuna*[2], *Aldo Merotto*[3] *and Martin Vila-Aiub*[4]*

[1] Department d'Hortofructicultura, Botànica i Jardineria, Agrotecnio-CERCA Center, Universitat de Lleida, Lleida, Spain, [2] Center for Scientific and Technological Research of Extremadura (CICYTEX), Agrarian Research Center "Finca La Orden" Valdesequera, Badajoz, Spain, [3] Department of Crop Science, Faculty of Agronomy, Federal University of Rio Grande Do Sul (UFRGS), Porto Alegre, Brazil, [4] Department of Ecology, IFEVA -CONICET, Faculty of Agronomy, University of Buenos Aires (UBA), Buenos Aires, Argentina

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University of Naples Federico II, Italy

****Correspondence:***
Martin Vila-Aiub
vila@ifeva.edu.ar

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Editorial on the Research Topic

Multiple Herbicide-Resistant Weeds and Non-target Site Resistance Mechanisms: A Global Challenge for Food Production

EVOLUTION OF MULTIPLE SURVIVAL MECHANISMS

The acquired inheritable trait of plants to survive and reproduce under herbicide exposure is defined as resistance. Herbicide resistance is an extraordinary example of adaptive evolution in weed species infesting agroecosystems with clear detrimental consequences on agriculture sustainability around the globe (Palumbi, 2001; Llewellyn et al., 2016). Multiple herbicide resistance is a compelling evolutionary process in which distinct survival mechanisms are present in a population or are combined within single plants, each endowing resistance to dissimilar site of action herbicides (Hall et al., 1994; Gaines et al., 2020). These multiple mechanisms may involve either target site (TSR) or non-target site resistance (NTSR) mechanisms or any combination endowing multiple resistance. Multiple resistance can evolve through unique events that sequentially select for resistance alleles within single plants and/or genetic exchange of independently evolved resistance mutations through pollen outcrossing among plants within or between populations. Regardless of the driving factor, the ultimate result is the stack of various distinct survival mechanisms at the plant and/or population level endowing broad resistance to multiple herbicides of dissimilar chemistries.

Genetic variability and reproductive biology of weed species are the most important factors that define the likelihood of multiple resistance evolution. *Lolium rigidum*, *Alopecurus myosuroides*, *Raphanus raphanistrum*, and *Amaranthus spp*. are among the weed species with the most remarkable ability to evolve multiple resistance through eco-evolution of TSR and NTSR mechanisms (Hall et al., 1997; Cocker et al., 1999; Walsh et al., 2004; Owen et al., 2014, 2015; Schultz et al., 2015; Han et al., 2016; Tétard-Jones et al., 2018). For instance, resistance due to reduced glyphosate and paraquat translocation co-evolving with an *ACCase* target site mutation has been identified in a single *L. rigidum* population (Yu et al., 2007), whereas other patterns of multiple resistance in this species reflect the presence of enhanced CYP-450 herbicide metabolism coexisting with *ACCase*, *ALS*, *α-tubulin*, and/or *EPSPS* point mutations

(Burnet et al., 1994a,b; Tardif and Powles, 1994; Neve et al., 2004; Han et al., 2016, 2021; Chen et al., 2018, 2020a). Another striking example of multiple resistance is found in *A. tuberculatus* populations where *PPO*, *ALS*, and *EPSPS* target site mutations have been identified co-evolving with enhanced metabolism of PSII and HPPD inhibiting herbicides (Schultz et al., 2015).

Novel resistance mechanisms in weeds have been identified recently although some were thought unlikely to evolve. For instance, glyphosate resistance is possible through aldoketoreductase (AKR)-based metabolism (Pan et al., 2019), up-regulation of an ABC membrane transporter pumping out glyphosate outside the cell (Pan et al., 2021) and programmed cell death causing rapid necrosis (Van Horn et al., 2018). Likewise, 2,4-D resistance due to either CYP-450 based metabolism (Giacomini et al., 2020), a double point mutation (Leclere et al., 2018) or 9-codon deletion in an auxin transcriptional repressor (Figueiredo et al., 2021), or rapid necrosis (De Queiroz et al., 2020) have also been reported. These recent findings highlight that herbicide selection for many survival mechanisms will occur and increase the chances for plants to harbor multiple resistance mechanisms. Multiple herbicide resistance highlights the concurrent dynamic spread of multiple resistance alleles in weeds which exposes a serious threat to productivity of current cropping systems.

RECENT ADVANCES IN NTSR MECHANISMS

Mechanisms that can contribute to NTSR are complex and involve several different gene types and families. This molecular and genetic complexity makes the identification of particular genes involved in NTSR difficult. Recent advances in this area have been the identification of putative NTSR genes contributing to enhanced herbicide metabolism (EHM).

The latest finding has been the elucidation for the first time that up-regulation of the AKR enzyme contributes to glyphosate resistance in *Echinochloa colona*, by degrading glyphosate to its metabolite, aminomethylphosphonic acid (AMPA; Pan et al., 2019). This discovery further supports results published in this Research Topic, showing glyphosate metabolism in an *E. crus-galli* population from Portugal (Vázquez-García, Rojano-Delgado et al.). The identification of CYP-450 genes (phase I) that can degrade herbicides from different sites of action (SoA) has been carried out recently. CYP81A subfamily has been shown to metabolize herbicides from at least five chemically unrelated groups, both in *L. rigidum* and *E. phyllopogon* (Dimaano et al., 2020; Han et al., 2021). Unraveling which SoA and chemical herbicide families individual CYPs can metabolize, and their identification in different R species could help predicting metabolic-based cross-resistance patterns and thus assist in chemical options for weed management practices.

CYP-450 has been shown to endow herbicide resistance in broadleaf weed species too, as reported for *Glebionis coronia* to ALS inhibitors in this Research Topic (Hada et al.). It is worth mentioning studies confirming that CYP-450 is involved in 2,4-D metabolism in *A. tuberculatus* (Figueiredo et al., 2018) and *Papaver rhoeas* (Torra et al., 2021). Moreover, in *P. rhoeas*, the same CYP-450 has been shown to confer cross-resistance to both 2,4-D and imazamox in several R populations (Torra et al., 2021).

Phase II herbicide metabolism mainly involves conjugation to GSH mediated by GSH S-transferases (GSTs). Metabolic resistance to VLCFA inhibiting herbicides such as flufenacet and pyroxasulfone in *Alopecurus myosuroides* and *L. rigidum* populations is possible due to enhanced GST-mediated metabolism *via* differentially expressed GSTs (Dücker et al., 2019, 2020; Goggin et al., 2021). In this Research Topic, empirical evidence of herbicide metabolism *via* CYP-450 is provided in three articles (Yanniccari, Gigón et al.; Chen et al.; Hada et al.), of GST in two (Wang et al.; Rangani et al.), and of both CYP-450 and GST in five studies (Scarabel et al.; Shyam et al.; Suzukawa et al.; Franco-Ortega et al.; Torra et al.).

All types of resistance mechanisms can get stacked in R plants, both TSR and NTSR, but also different genes conferring EHM. Several studies have reported over-expression of many genes in NTSR plants compared to S ones, also including those encoding for degrading enzymes such as CYP-450 and GST (Gaines et al., 2020). However, this does not necessarily imply a process of recurrent selection and the concomitant slow accumulation of metabolic resistance genes in a R population. There is evidence that differentially expressed genes responsible for EHM could be under genomic co-expression clusters or across long chromosomal intervals (Giacomini et al., 2020). One major implication of this clustering is the likelihood of a shared mechanism of gene regulation for these regions with NTSR genes. Therefore, potentially, a single gene, that is, a single resistance mechanism, could be responsible of the reported over-expression of several genes involved in EHM and NTSR.

In this Research Topic, Franco-Ortega et al. suggested that plant responses to biotic and abiotic stressors are integrally linked to NTSR-based herbicide resistance mechanisms. Regulation of gene expression involved in stress-response and NTSR is probably a complex process but may include herbicide-responsive genes. Recently, HPPD-inhibiting herbicide responsive genes have been found in *A. tuberculatus*, with little overlap in gene expression patterns between R and S genotypes bringing out dynamic differences in response to herbicide treatment (Kohlhase et al., 2019). Similarly, a contributing article in the present Research Topic, points out that S-metolachlor (VLCFA inhibitor) can further increase the expression of two GSTs in R plants (Rangani et al.).

Differential herbicide translocation between S and R plants constitutes another set of NTSR mechanisms. Membrane carrier proteins (ABC family) are already being unveiled and suggested to be involved in phase III of EHM (Gaines et al., 2020). Although reduced glyphosate translocation was described as a resistance mechanism long ago, only recently the first glyphosate cell membrane carrier has been identified (ABCC-type transporter) conferring glyphosate resistance in *E. colona* (Pan et al., 2021). Active root exudation as a NTSR mechanism has been recently reviewed by Ghanizadeh and Harrington (2020). This mechanism could contribute to imazamox resistance in *Euphorbia heterophylla* (Rojano-Delgado

et al., 2019) and MCPA resistance in *Raphanus raphanistrum* (Jugulam et al., 2013).

Rapid Necrosis: An Intriguing Mechanism of Herbicide Resistance

A fast and localized effect of glyphosate and 2,4-D has been identified in *Ambrosia trifida* (Brabham et al., 2011) and *Conyza sumatrensis* (De Queiroz et al., 2020). This phenomenon has been called rapid necrosis (RN), and was primarily proposed as Phoenix resistance (Gressel, 2009) as apparent "dead" plants were able to regrow a few days after herbicide treatment. The physiological basis of this surviving mechanism is unknown and thus, the classification of RN as TSR or NTSR is difficult. The RN caused by 2,4-D may be related to defective Aux/IAA repressors, TIR1/AFB receptors and ARF transcription factors and in that case would be classified as TSR since these proteins are directly related to the 2,4-D action. Exogenous application of aromatic amino acids decreased RN in *A. trifida* caused by glyphosate (Moretti et al., 2018), indicating a potential TSR mechanism of resistance.

In both 2,4-D and glyphosate cases, a potential reduced herbicide translocation resistance mechanism could be related to ABC transporters (Pan et al., 2021), however, alterations in translocation and cell exclusion resulting in 2,4-D and glyphosate resistance were not identified with the RN phenotype. Some evidence suggests that programmed cell death may be caused not only by pathogens as originally discovered but also triggered by other biotic and abiotic stresses such as herbicides (Burke et al., 2020). Several studies have reported the influence of environmental effects on the occurrence and variability of RN (Harre et al., 2018; De Queiroz et al., 2020), which highlight the difficulties of studying RN under the variable conditions found in the field and experimental conditions. Distinguishing the biochemical processes that cause RN from those that are the consequence of RN is needed to better understand this intriguing herbicide resistance mechanism.

Contributions in the Research Topic

Contributions in this Research Topic reported both TSR and NTSR mechanisms. Eight out of 13 articles reported mechanisms of TSR nature (all substitutional mutations), which in some cases can confer cross-resistance to different herbicide chemistries within the same SoA (Scarabel et al.; Vázquez-García, Alcántara-De La Cruz et al.; Yanniccari, Gigón et al.; Hada et al.; Torra et al.). Among these contributions, we shall highlight those reporting multiple-resistance through the accumulation of several substitutional point mutations in different herbicide target enzymes involving ALS, ACCase and EPSPS inhibitors (Scarabel et al.; Vázquez-García, Alcántara-De La Cruz et al.).

In relation to NTSR mechanisms, three contributions reported about herbicide differential absorption and translocation (Suzukawa et al.; Vázquez-García, Rojano-Delgado et al.; Yanniccari, Vázquez-García et al.), whereas most of them (11 out 13) documented cross-resistance due to some level of EHM. It is also remarkable that seven contributions demonstrated the co-evolution of TSR and NTSR mechanisms at both plant and population level.

Resistance to ALS, ACCase, and EPSPS inhibiting herbicides are the most reported cases in this Research Topic, with 8, 7, and 5 contributions, respectively, which agrees with the SoA herbicides most related to herbicide resistance worldwide (Heap, 2021). Resistance to pre-emergence herbicides in different cropping systems is reported, as multiple resistance in combination to the three previously mentioned post-emergence SoA herbicides. Resistance to microtubule assembly (Suzukawa et al.; Chen et al.; Franco-Ortega et al.), VLCFA (Suzukawa et al.; Rangani et al.; Torra et al.), PSII (Shyam et al.; Franco-Ortega et al.; Torra et al.), synthetic auxins (Shyam et al.; Suzukawa et al.; Franco-Ortega et al.; Torra et al.), and both PPO and HPPD in a single six-way-resistant Palmer amaranth (*Amaranthus palmeri*) population (Shyam et al.) are contributions in this Special Issue.

Ten out of 13 contributions reported on herbicide resistance in grass weed species, and three in broadleaf weeds. *Lolium* ssp. is the most reported genus in the Research Topic (six articles), followed by *A. myosuroides* and *Bromus* ssp. (2), and *E. crus-galli* and *Beckmannia syzigachne* (1). Among dicots, two articles reported on the global invasive weed species *A. palmeri* (Shyam et al.; Rangani et al.) and one contribution on *Glebionis coronaria* (Hada et al.).

CURRENT RESEARCH GAPS AND PROSPECTS

(1) Multiple herbicide resistance may result from co-evolution of both NTRS and TSR mechanisms (Vila-Aiub et al., 2005; Powles and Yu, 2010; Bostamam et al., 2012; Gherekhloo et al., 2017; Peterson et al., 2018; Cao et al., 2021). An intriguing question is the evolutionary and ecological consequences of the interaction between NTSR and TSR mechanisms in protecting single plants from herbicide damage (Raymond et al., 1989). For instance, point resistance mutations co-existing with up-regulation of herbicide metabolism (EHM by CYP-450 or GST), both endowing resistance to herbicides targeting the same SoA are ubiquitous in resistant weeds (Tardif and Powles, 1994; Chen et al., 2020a,b). Do these resistance mechanisms combine their effects on plant protection in an additive or multiplicative mode? Would it be possible for a single mechanism to endow the maximum protection level making the addition of a second mechanism an ecological redundancy?

(2) Improved understanding of the biology of plant systems will benefit the understanding of gene regulation of NTSR and the effects of environmental factors on the evolution of herbicide resistance. Further studies related to epigenetic regulation caused by direct or indirect herbicide effects will further increase our understanding of herbicide resistance. The NTSR mechanisms associated with EHM are dependent on a complex gene regulation and we are currently just discovering the final players of a large network. Advances on CYP-450 and GST gene identification as well as their regulation and crystallographic

information will reveal a fascinating environmental-plant-herbicide interaction system.

(3) Current recommendations for pesticide resistance prevention are based on rotation and mixing of different SoA pesticides (Bourguet et al., 2013; Baym et al., 2016). However, rotation and/or mixing of herbicides resulting in a similar selection pressure for a particular resistance mechanism (e.g., EHM) will increase the risk of resistance evolution (Comont et al., 2020). The advances in the knowledge of NTSR mechanisms will be necessary for making resistance management decisions involving the use of herbicides targeting different metabolic networks, assuming it is possible to avoid development of some of these resistance mechanisms by modifying management.

AUTHOR CONTRIBUTIONS

All authors conceived and wrote this Editorial contribution for the Research Topic *Multiple Herbicide-Resistant Weeds and Non-target Site Resistance Mechanisms: A Global Challenge for Food Production*.

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Pro-197-Ser Mutation in *ALS* and High-Level GST Activities: Multiple Resistance to ALS and ACCase Inhibitors in *Beckmannia syzigachne*

Jingjing Wang[†], *Jingchao Chen*[†], *Xiangju Li*, *Dan Li*, *Zheng Li* and *Hailan Cui*[*]

Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

American sloughgrass (*Beckmannia syzigachne* Steud.) is one of the most troublesome weeds infesting wheat and canola fields in China. Some biotypes cannot be controlled, either by acetolactate synthase (ALS) or acetyl coenzyme A carboxylase (ACCase) inhibitors, which are the main herbicides for controlling this weed. However, very few studies have investigated multiple resistance mechanism in *B. syzigachne*. In this study, a *B. syzigachne* biotype with a high resistance to ALS inhibitors we have reported was also showed relatively lower resistance to ACCase inhibitors, with a resistance index around 7. RNA-seq analysis was used to investigate the factors responsible for multiple resistance, and 60,108 unigenes were assembled by *de novo* transcriptome assembly and then annotated across eight databases. A Pro-197-Ser mutation was identified in the *ALS* gene by SNPs analysis and validated by PCR, while no mutation was identified in the *ACCase* gene. Nineteen candidate metabolic genes were screened and their overexpression was confirmed by qPCR. The expression of *GST-T3* and *GST-U6* in resistant plants ranged from 7.5- to 109.4-folds than that in susceptible ones at different times after two kinds of herbicide treatment. In addition, GST activities in resistant plants were 3.0–5.0 times higher than that in susceptible plants. Other novel resistance factors also showed high correlation with multiple resistance which included four genes encoding disease resistance proteins, a transcription factor (*MYC3*), and one gene conferring blight resistance. In this research, a *B. syzigachne* biotype was confirmed to have evolved multiple resistance to ACCase and ALS inhibitors. The Pro-197-Ser mutation in *ALS* gene and high-level GST activities were confirmed responsible for the multiple resistance. Characterized disease-resistance proteins, transcription factor, and blight-resistance proteins may play an essential role in these multiple herbicide resistance.

Keywords: herbicide resistance, weeds, metabolism, RNA-seq, overexpression

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Federal University of São Carlos, Brazil
Hugo Enrique Cruz-Hipolito,
Bayer, Puerto Rico

***Correspondence:**
Hailan Cui
cuihailan413@163.com

[†]*These authors have equally contributed equally to this work*

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INTRODUCTION

American sloughgrass (*Beckmannia syzigachne* Steud.) is a troublesome diploid weed (2n = 14), which threatens many crop varieties in China such as wheat (*Triticum aestivum* L.) and oilseed rape [*Brassica rapa* L. subsp. Oleifera (DC.) Metzg.] (Rao et al., 2008; Li et al., 2015; Du et al., 2016; Li et al., 2017). Inhibitors of acetyl coenzyme A carboxylase (ACCase; EC 6.4.1.2) and acetolactate synthase (ALS; EC 4.1.3.18) are the main herbicides that are used to control the *B. syzigachne* in China. However, after many years of selection pressure, this weed evolved cross- or multiple-resistance to these two mechanisms of herbicides (Li et al., 2013; Li et al., 2015; Li et al., 2017). Resistant *B. syzigachne* can survive under high doses of these two kinds of herbicides *via* a variety of mechanisms. We sought to investigate resistance mechanisms useful for its control.

Based on the confirmed resistance against the ACCase-inhibiting and ALS-inhibiting herbicides in weeds, two kinds of resistance mechanisms can be summarized; these are target-site resistance (TSR) and non-target-site resistance (NTSR) (Délye, 2005). TSR is caused by amino acid substitutions in the conserved regions or due to differences in the expression of target enzyme genes, and has been well described (Délye, 2005; Powles and Yu, 2010). A mutation in the carboxyl-transferase (CT) domain of *ACCase* causes weed resistance to ACCase-inhibitors and confers distinct cross-resistance patterns (Powles and Yu, 2010; Scarabel et al., 2011; Beckie and Tardif, 2012; Kaundun, 2014). With respect to *ALS*, an amino acid substitution is commonly found at eight positions, and the different mutations also result in different levels of resistance against this kind herbicide (Yu and Powles, 2014b; Heap, 2019). NTSR is complicated and commonly results due to the overexpression of a set of genes that lead to reduced herbicide absorption, translocation, or increased metabolism and sequestration (Délye, 2013). To date, many well-established gene families are known to be involved in NTSR, such as the cytochrome P450 monooxygenase (P450s), and glutathione S-transferase (GSTs) families (Yuan et al., 2007). Many genes of these families are confirmed to have conferred resistance in herbicide-resistant weeds (Iwakami et al., 2014; Saika et al., 2014; Iwakami et al., 2019).

It has been reported that the mechanism governing the resistance of *B. syzigachne* to ACCase-inhibiting herbicides mainly involves TSR, in which amino acid substitutions have been identified at six positions (1781, 1999, 2027, 2041, 2078, and 2096) in resistant *B. syzigachne* (Du et al., 2016; Liu et al., 2019). Only the mutation involving Pro197Ser was found in the *B. syzigachne* population resistant to ALS-inhibiting herbicides (Li et al., 2015; Wang et al., 2018). For NTSR, metabolic genes related to resistance have been reported in fenoxaprop-P-ethyl-resistant *B. syzigachne* (Pan et al., 2016a; Pan et al., 2016b). However, to our knowledge, no studies have reported the involvement of genes in the NTSR mechanisms in *B. syzigachne* with ALS resistance, though the NTSR mechanisms have been confirmed (Wang et al., 2018; Wang et al., 2019; Bai et al., 2020). Furthermore, resistance genes relative to multiple resistance to ACCase and ALS-inhibiting herbicide have not been reported. Recently, we found a *B. syzigachne* biotype with ALS-inhibiting herbicide resistance which confirmed in our previous study was also hardly controlled by the ACCase inhibitors (Wang et al., 2018).

Hence, this research aimed to (1) determine the multiple resistance levels of *B. syzigachne* to ALS- and ACCase-inhibitors, (2) explore the multiple resistance factors using next generation sequencing methods, (3) confirm the Single Nucleotide Polymorphisms (SNPs) or the expression for the candidate genes, and (4) determine the enzyme activity which relative to the multiple resistance.

MATERIALS AND METHODS

Plant Material and Whole Plant Assays

Resistant (R) *B. syzigachne* biotype were collected from Wuxi city (WC1148) and found evolved high-level resistance to ALS inhibitors in our previous study (Wang et al., 2018). A susceptible population (S) (WC1004), with no history of herbicide use was collected from near the R biotype. The plants of these two populations were cultured using the methods described in a previous study and thinned to 10 individuals per pot (Wang et al., 2018). Three kinds of ACCase inhibitor herbicide were selected for resistance assays (**Table S1**). The herbicides were applied when the seedlings reached the 3-leaf stage using a spray chamber with a moving TeeJet® XR8002 flat fan nozzle under a pressure of 0.275 MPa (Wang et al., 2018). Dry weights of the shoots (dried at 80°C for 48 h) in each pot were measured 21 days after treatment (DAT). The experiment of whole plant assay was repeated twice in a completely randomized design that had three biological replicates (three pots per treatment).

Sample Collection and RNA Extract for RNA-Seq

The plant culture conditions for the R and S populations were the same as in previous reports (Wang et al., 2018). The experimental design included three different treatment for R and S: without herbicide treatment control (CK), mesosulfuron-methyl treatment (M), and fenoxaprop-P-ethyl treatment (F). When the individuals reached the 3–5 leaf-stage, each biotype was treated with 70 g a.i. ha^{-1} of fenoxaprop-P-ethyl and the 20 g a.i. ha^{-1} mesosulfuron-methyl, respectively. After 24 h, the leaf tissues of three biological replicates were random collected, and also the samples without herbicide treatment were collected as controls with three individuals for each biotype. All the 18 samples were snap frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the RNAprep Pure Plan Kit (Tiangen Biotech Beijing CO., LTD, Beijing, China). The degradation and contamination were checked by 1.0% agarose gel electrophoresis. RNA purity and concentration were determined using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The RNA integrity was assessed by the Agilent Bio analyzer 2,100 system (Agilent Technologies, Palo Alto, USA) (Chen et al., 2017a).

Construction of cDNA Libraries, Sequencing, and Bioinformatics Analysis

The cDNA library construction was performed by a common method using the 18 detected RNA samples by Allgwegene Health Co. (Beijing, China) (Chen et al., 2017a). Paired-end reads (125 bp) were determined using an Illumina HiSeq™ 4000 (Illumina Inc., San Diego, CA, USA) platform, and clean reads were obtained from which raw reads with low quality were removed. Subsequently, *de novo* transcriptome assembly was carried out using the Trinity platform and the longest transcript of each locus was termed a "unigene" for subsequent annotation (Grabherr et al., 2011). Eight databases were selected for the annotation of the unigenes (**Table S2**). The software BLASTX (E-value < 10^{-5}), BLAST2GO, and Blastall were used to predict and classify the different unigenes, as in previous studies (Tatusov et al., 2001; Ana et al., 2005; Clark et al., 2007).

SNPs Analysis for Target Genes and PCR Validation

For SNP calling, picard tools was used to map the paired-reads from each sample to the reference genome sequence of *Brachypodium distachyon* [L.] Beauv. The software GATK2 was used for SNP discovery and the detection stringency conditions include at least five reads calling the variant and >30 mapping quality score (Mckenna et al., 2010). To confirm the SNPs results for *ALS* and *ACCase* gene, the PCR method was selected to investigate the mutations in these two genes. DNA of the leaf tissues was exacted (15 individuals both for R and S populations) using the Hi-DNAsecure Plant Kit (DP350) by following the manufacturer's instructions (Tiangen Biotech Beijing CO., Ltd., Beijing, China). Two pairs of PCR primers were design to clone *ALS* (1F-5'CGCCTTACCCAAACCTACT3', 1R- 5'ATGCGGCTGCTTG TTCTT3'; 2F- 5'ATCCCACCACAATATGCTATCC3', 2R-5'TCACAGTTGACCACACTTC3'); the amplification products of these two pairs of primers were 1035 and 766 bp in size, respectively, which encompass all of the reported mutation sites. One pair of primers was designed (F-5'AAACTCTGGT GCTCGGATTG3'; R-5'TAGGCTTCCATTTGCTCCC3') to clone *ACCase* containing the CT region, and the PCR products were 1308 bp in size. The PCR mixture components and amplification conditions were in accordance with those reported in a previous study (Chen et al., 2017b), and the annealing temperature for all the primer pairs was 58°C. After confirming fragment amplification using 1% agarose gels prepared in 1× TAE, followed by staining with ethidium bromide, the PCR products were sequenced by Tsingke (Tsingke Biological Technology, Beijing Co., Ltd., Beijing, China), and the *ALS* and *ACCase* sequences of all the populations were aligned using the software DNAMAN (Lynnon BioSoft, San Ramon, CA, USA). The experiment of PCR was done twice and had 15 biological replicates both for R and S biotypes.

Analysis of Differentially Expressed Genes

Expression analysis was performed for the selected 18 samples, which were same as described above, and the samples were divided into different groups. The samples of the R and S biotypes without herbicide treatment were named CK1148 and CK1004, respectively. The sample groups treated with mesosulfuron-methyl for the R and S biotypes were named M1148 and M1004, respectively. Similarly, the samples groups treated with fenoxaprop-P-ethyl for the R and S populations were named F1148 and F1004, respectively. The cDNA library constructs and the clean reads detected for the 18 samples were the same as described above. The clean reads for the 18 libraries were mapped back onto the reference transcriptome, which was assembled in the above part by Bowtie 2 v.2.2.3 (Li and Dewey, 2011). The expression of each gene between sample pairs (CK1148 vs. CK1004, F1148 vs. CK1148, M1148 vs. CK1148, F1004 vs. CK1004, M1004 vs. CK1004, F1148 vs. F1004, and M1148 vs. M1004) were analyzed both by the numbers of reads and fragments aligned per thousand bases per million reads (FPKM), which was conducted using DESeq 2 v.1.4.5 (Audic and Claverie, 1997; Love et al., 2014). The false discovery rate is considered to be a standard to confirm the threshold of *P* values in multiple tests and analyses (Benjamini and Yekutieli, 2001).

Candidate Resistance Gene Selection and qPCR Validation

Candidate resistance genes were selected considering the reported NTSR genes include metabolism and signaling functions genes, the statistical significance ($q < 0.05$), and magnitude of differences in expression between the above treatment groups ($|\log_2 (\text{fold change})| \geq 1$). quantitative Real-time PCR (qPCR) was performed to confirm the accuracy of their expression. The best reference gene was selected from among capsine phosphatase (*CAP*), glyceraldehyde 3-phosphate dehydrogenase (*CADP*), and ubiquitin (*UBQ*) by the software BestKeeper (Pfaffl et al., 2004). The plant culture method, herbicide treatment for the individuals, and sample selection and storage were the same as described above. Total RNA was exacted, and first strand cDNA synthesis was performed (Tiangen Biotech Co., Ltd., Beijing, China) for all samples, and then primers were designed for all the genes (reference genes and the candidate resistance genes) using the software Oligo 7.0 (**Table S3**). The qPCR reaction was performed in 25 μL reaction volumes on an ABI 7500 PCR instrument under common conditions (Chen et al., 2017a). Relative expression of the selected genes was quantified using the $2^{-\Delta\Delta}$Ct method (Bustin et al., 2009). For the qPCR, three biological replicates were performed for each treatment, and three technical replicates was done for each sample.

Expression and Enzyme Activity Validation of GSTs

The plant culture for the R and S biotypes, and the herbicide treatment were same as described above. At 0, 24, 48, and 72 h, leaf samples for each treatment (at least three replicates) were collected and stored at -80°C. Total RNA was exacted and first strand cDNA synthesis was performed using the methods described above for all samples. The expression levels of two GSTs-related genes (*GST-T3* and *GST-U6*) were detected using the qPCR method for all the samples, respectively, and the GST

activity for the samples were also detected using a Glutathione S-transferase Microplate Assay Kit (CAK 1047) following the manufacturer's instructions (Cohesion Biosciences Co., Ltd., Suzhou, China). Each sample (0.1 g leaf tissues) was assayed at 340 nm using a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). The results were expressed as U g^{-1}, and one unit of GST activity was defined as the enzyme generating 1 μmol of glutathione and 1-chloro-2, 4-dinitrobenzene conjugate in the reaction time. Both the gene expression and GST activity assay were repeated twice and at least three biological replicates were performed for each treatment.

Statistical Analysis

The values of herbicide doses required to reduce 50% of the plant growth (GR_{50}) were estimated through nonlinear regression with a log-logistic model:

$$Y = y^0 + \frac{a}{1 + \left(\frac{X}{X_0}\right)^b}$$

using Sigma Plot 12.0 (Systat software, San Jose, CA, USA) (Seefeldt et al., 1995). In this model, b is the slope of the curve, y_0 is the lower limit, a is the difference between the upper limit and the lower limit, and X_0 is the GR_{50}. Resistance levels were determined by calculating the ratio of the GR_{50} of the resistant biotype to that of the susceptible biotype, and the results are expressed as resistance index (RI). The difference of gene expression and GST activity between R and S biotypes were analyzed by the Student's t-test, which were performed with SPSS 13.0 (SPSS, Chicago, USA).

RESULTS

Resistance Levels to ALS- and ACCase-Inhibiting Herbicides

The sensitivity of the R (WC1148) and S (WC1004) biotypes in response to four different ACCase inhibitors (fenoxaprop-P-ethyl, clodinafop-propargyl, clethodim, and pinoxaden) was calculated. The GR_{50} values of the S biotype WC1004 were 42.4, 12.1, 11.4, and 11.8 g a. i. ha^{-1}, respectively. For the R biotype WC1148, the GR_{50} values were 87.0, 39.4, 84.5, and 89.2 g a. i. ha^{-1}, respectively. Compared with those of the sensitive biotypes, the RI of WC1148 were 2.1, 3.3, 7.4 and 7.5, respectively (**Table 1**). These results indicated that the ALS inhibitors resistant biotype WC1148 evolved multiple resistance to the ACCase inhibitors.

RNA-Seq and *De Novo* Assembly, Gene Annotation, and Functional Classification

The OD_{260}/OD_{280} values (around 2.0) and the RNA integrity number (RIN) values (>6.0) indicated that the RNA integrity of the samples was suitable for the requirements of subsequent experiments. The cDNA library of 18 RNA samples were analyzed: 449,583,465 raw reads, and 436,188,739 clean reads which ranged from 22,724,569 to 24,516,824 per sample were generated, respectively (**Table S1**). We obtained 118,111 unigenes that ranged from 201 to 21,671 bp in size and with an N50 of 1,328 bp.

More than half (50.89%) of the total 118,111 unigenes were successfully annotated in at least one database, and 8,197 unigenes were annotated in all of the database (**Table S1**). 35,616 unigenes were assigned into 42 functional categories by the Gene Ontology (GO) database and including 20 for "biological process", 12 for "cellular component", and 10 for "molecular function" (**Table S4**). In total, 25,598 unigenes were categorized into 26 Clusters of Orthologous Groups of proteins (KOG) classifications (**Table S5**). The 7,893 assembled sequences were mapped to the reference canonical pathway in Kyoto Encyclopedia of Genes and Genomes (KEGG) (**Table S6**). The 48,600 unigenes were assigned putative annotations by the NCBI non-redundant protein sequences (NR), 39,869 unigenes by NCBI non-redundant nucleotide sequences (NT), 31,291 unigenes by a manually annotated and reviewed protein sequence database (SwissProt), and 39,310 unigenes in the Protein family (Pfam) database.

SNPs Analysis and PCR Validation for *ALS* and *ACCase* Gene

Two SNPs were detected for the *ALS* gene (c38811_g1) using the RNA-seq analysis, while, no SNPs was found for the *ACCase* gene (**Table S7**). For the *ALS* gene, one SNP was only found in the resistant individuals (C to T at position 629) which causing the amino acid Pro change to Ser (**Table S7**). While, another SNP (G to A at position 820) was only found in the susceptible individuals but without causing any amino acid change. To

TABLE 1 | GR_{50} values and resistance index of *B. syzigachne* to ACCase inhibitors in the R and S biotypes.

Classification of herbicide[a]		Herbicides	Biotypes[b]	GR_{50} ± SE (g a.i.ha^{-1})	P-value	Resistanceindex (RI)[c]
ACCase inhibitors	APPs	Fenoxaprop-P-ethyl	S	42.4 ± 9.8	<0.001	—
			R	87.0 ± 24.8	<0.001	2.1
		Clodinafop-propargyl	S	12.1 ± 9.2	<0.001	—
			R	39.4 ± 7.8	<0.001	3.3
	CHDs	Clethodim	S	11.4 ± 5.7	<0.001	—
			R	84.5 ± 45.5	<0.001	7.4
	PPZs	Pinoxaden	S	11.8 ± 3.6	<0.001	—
			R	89.2 ± 65.9	<0.001	7.5

[a]*APPs, Aryloxyphenoxypropionates; CHDs, Cyclohexanediones; PPZs, Phenylpyrazoline;*
[b]*S: susceptible biotype; R: resistant biotype; GR_{50}: herbicide doses required to inhibit dry weight by 50% compared to untreated controls;*
[c]*RI, resistance index, and determined by dividing the GR_{50} of the R by that of S biotype.*

confirm the results of the SNPs assay, parts of the *ALS* and *ACCase* genes which contain all the common mutation sites of these two kinds of inhibitors in *B. syzigachne* were cloned from the R and S populations. Comparing the conserved regions of the *ALS* gene for the S and R biotypes, it was found that 197 sites of *ALS* were mutated from proline CCC (Pro) to serine TCC (Ser), and no other mutation sites were found in any of the 15 individuals (**Figure 1**). By comparing the *ACCase* CT region of the R and S biotypes, no mutation was found in the CT region of the *ACCase* gene. The results of the chromatograms show that the *ALS* gene in the R biotypes was a single peak at the mutation site, indicating a homozygous mutation (**Figure 1**), and the detected 15 individuals of R biotypes showed the same mutation type.

Analysis of Differentially Expressed Genes

Seven different comparison combinations were designed as the biotype WC1148 showed multiple resistance to ALS and ACCase inhibitor herbicides (**Figure 2**). Differentially expressed genes between these groups ($|\log_2$ (fold change)$| \geq 1$ and $q < 0.05$) were selected as candidate resistance genes. The number of up- and down-regulated genes in CK1148 vs CK1004 combination was 700 and 907, respectively. For the group set of M1148 vs CK1148, and F1148 vs CK1148, the number of up- and down-regulated genes was no more than 300. After herbicide treatment, differentially expressed genes between the R and S biotypes (M1148 vs. M1004 and F1148 vs. F1004) were more than 400. The number of up- and down-regulated genes in the M1004 vs. CK1004 combination was 2,720 and 4,185, respectively. However, the number of up- and down-regulated genes in the F1004 vs. CK1004 combination was 162 and 233, respectively (**Table 2**).

Selection of Candidate Non-Target Site Resistance Genes

We analyzed and selected factors related to NTSR based on the reported mechanism in weeds (Délye, 2013; Yu and Powles, 2014a; Pan et al., 2016a). The reported genes relative to the metabolic enzyme families in *B. syzigachne* were considered which include *CytP450*, *GST*, *ABC* transporter family, and esterase gene families (Pan et al., 2016a). Other genes up-regulated in all the comparative groups were considered, especially for up-regulated genes in CK1148 vs. CK1004. After preliminary analysis of the different expression of the genes between different treatment groups, 19 candidate genes were related to resistance. Among these candidate genes, common metabolic genes were identified, which included three genes annotated as GSTs, one gene being an ABC transporter, two protein kinase genes, one annotation as UDP-glycosyl transferase, and two for oxidases. In addition, certain novel resistant factors were found, which included two immune proteins, five disease resistance proteins, two termed 'other' transferase, and one transcription factor (**Table 3**).

qPCR Validation of Candidate Metabolic Resistance Gene Expression

The expression of the selected contigs were confirmed using the qPCR method, and UBQ was the best reference gene which analyzed by Bestkeeper (**Table S8**) (Pfaffl et al., 2004). The expression levels of 13 candidate resistance genes were validated by qPCR at 0 and 24 h after treatment with 20 g a.i. ha^{-1} mesosulfuron-methyl for sensitive R and S biotypes. The

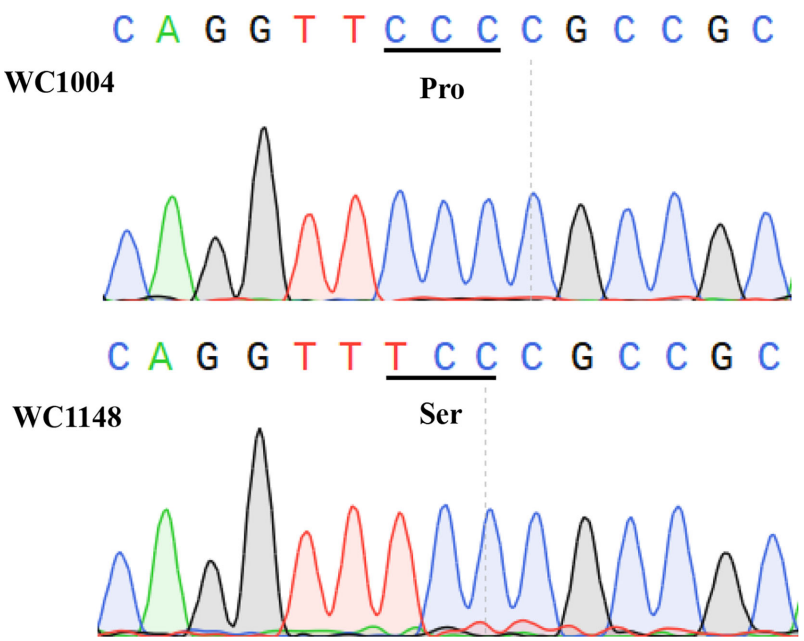

FIGURE 1 | Sequences result for *ALS* genes in WC1148 and WC1004 biotypes, respectively. A Pro-107-Ser mutation in the *ALS* gene evolved in the resistant biotype WC1148, and DNA sequencing chromatograms show the homozygous mutation.

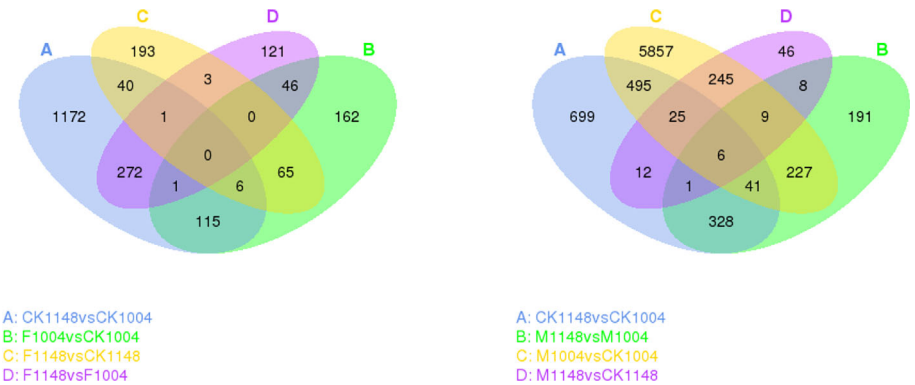

FIGURE 2 | Venn diagram showing differential gene expression between the different group sets for the resistant biotype WC1148 and susceptible biotype WC1004. The circle represents the comparisons, the sum of the numbers in each circle represents the genes showing differential expression, and the overlapping part of the circle represents the number of the same genes between different comparisons.

TABLE 2 | Number of differentially expressed genes among different groups.

Treatment groups	Differentially expressed gene	Up-regulated	Down-regulated
CK1148 vs. CK1004	1607	700	907
M1148 vs. CK1148	352	274	78
F1148 vs. CK1148	308	115	193
M1004 vs. CK1004	6905	2720	4185
F1004 vs. CK1004	395	162	233
M1148 vs. M1004	811	393	418
F1148 vs. F1004	444	203	241

CK: untreated herbicides; M: mesosulfurn-methyl treatment; F: fenoxaprop-p-ethyl treatment.

TABLE 3 | Candidate genes related to mesosulfuron-methyl and fenoxaprop-p-ethyl metabolism in *B. syzigachne*.

Gene ID	Gene annotation	Fold-change(F1148 vs F1004 or M1148 vs M1004)	Padj
c59327_g1	GST-T3	8.68	2.22×10^{-20}
c9871_g1	GST-U6	4.82	2.62×10^{-20}
c81158_g1	GST-U6	5.46	2.05×10^{-3}
c61256_g2	ABC G family member 15	5.81	3.57×10^{-20}
c52253_g1	UDP-glycosyltransferase 88F5	6.57	6.51×10^{-20}
c30288_g1	LRR RLK At4g08850	8.36	1.60×10^{-9}
c64517_g5	LRR RLK At1g56140	3.95	8.94×10^{-3}
c61823_g3	Iron/ascorbate family oxidoreductases	6.82	9.95×10^{-7}
c50888_g2	L-pipecolate oxidase	4.03	3.25×10^{-3}
c61143_g4	Late blight resistance protein R1B-16	10.86	6.83×10^{-20}
c71182_g1	Late blight resistance protein R1A-10	7.76	2.59×10^{-4}
c41866_g1	Disease resistance protein At1g15890	10.52	7.67×10^{-13}
c62003_g3	Disease resistance protein At1g58602	9.99	4.94×10^{-10}
c63887_g5	Disease resistance protein RPP13	5.33	1.10×10^{-6}
c64184_g2	Disease resistance protein RGA3	4.30	1.15×10^{-4}
c51431_g2	Disease resistance protein RPP13	7.11	5.85×10^{-4}
c59149_g1	Polyamine aminopropyltransferase	5.99	1.40×10^{-10}
c45597_g1	Anthranilate N-benzoyltransferase protein 1	6.79	6.83×10^{-4}
c96180_g1	Transcription factor MYC3	3.77	4.11×10^{-2}

results showed that in the absence of treatment with the ALS inhibitor mesosulfuron-methyl, seven genes, including *GST-U6*, *disease resistance protein At1g4809*, and *disease resistance protein At1g58062* in the R biotype, exhibited 5.8-1124.0 times higher expression than that in S (**Figure 3**). However, other genes show similar expression level between the R and S biotypes (**Figure 3**). After this herbicide treatment, all these candidate genes showed 5.9–4928.2 times higher expression levels in the R biotype than that in the S biotype.

Moreover, eight candidate resistance genes were validated by qPCR at 0 and 24 h after treatment with 70 g a.i. ha^{-1} fenoxaprop-p-ethyl. Among these genes, six showed higher

expression in the R biotype both before and after herbicide treatment than that in S biotype. In particular, for the gene *disease resistance protein RPP13*, without herbicide treatment, the expression level in the R biotype was 300 times higher than in the S biotype (**Figure 3**). After herbicide treatment, the expression could reach 700 times higher.

Expression and Enzyme Activity Validation of GSTs

The expression levels of two unigenes (*GST-T3*, *GST-U6*) annotation to GSTs were analyzed at different times after herbicide treatment. The results show that expression of these two genes in the R individuals was higher than that in the S individuals at each time point under herbicide treatment (**Figure 4**). After mesosulfuron-methyl treatment, the expression of *GST-T3* in R can reach to 53.8-folds than S, similar results (108.5-folds) were also found after fenoxaprop-P-ethyl treatment. Without herbicide treatment, the expression of *GST-U6* was 46.5 times higher than in S and show slight decreased after these two kinds herbicide treatment.

To determine whether the higher expression of these genes of GSTs increase the GST active in R biotype. GST activity was investigated in both R and S biotypes at 0, 24, 48, and 72 h after mesosulfuron-methyl and fenoxaprop-P-ethyl treatment. The results show that in R plants without herbicide treatment, the GST activity was 3.4 U g^{-1}, which was 3.0 times higher than that in the S plants (**Figure 5**). After mesosulfuron-methyl treatment, GST activities were slightly increased in R biotype and reached a peak at 48 h (4.6 U g^{-1}), which was 5.0 times higher than that in S biotype. However, it was slightly decreased in the S biotype, which is around 0.8 U g^{-1}. Similar results were also found in these two biotypes after fenoxaprop-P-ethyl treatment. The GST activities were increased in R biotype and can reach to 4.9 U g^{-1}, while GST activities in the S biotype was around 1.0 U g^{-1} at all the time points. GST activities in the R plants were significantly higher than those in the S plants at all the time points.

DISCUSSION

Multiple Resistance Levels to ALS and ACCase Inhibitors

In this study, we confirmed that a *B. syzigachne* biotype had evolved multiple resistance to ALS and ACCase inhibitor herbicides. In our previous research, this biotype showed higher resistance to mesosulfuron-methyl, and also exhibited higher cross-resistance to other ALS inhibitor herbicides, such as flucarbazone, imazapic, pyroxsulam, and pyribenzoxim (Wang et al., 2018). In this study, the same biotype shows relatively low resistance levels to the ACCase inhibitor herbicide, with resistance levels ranging from 2.1 to 7.5. Cross-resistance is a common phenomenon in weed species resistant to ALS and ACCase inhibitor herbicides, such as *Conyza canadensis* (Zheng et al., 2011), *Myosoton aquaticum* L. (Liu et al., 2015), and *Bromus tectorum* L. (Park and Mallory-Smith, 2004). *Beckmannia syzigachne*, biotypes with cross-resistance to ACCase and ALS inhibitor herbicides are frequently reported in China (Li et al., 2013; Li et al., 2014; Li et al., 2015; Pan et al., 2015; Tang et al., 2015; Du et al., 2016; Liu et al., 2019; Wang et al., 2019). In addition, a biotype was also reported to have evolved multiple resistance to these kinds of herbicide (Li et al., 2017). In contrast to the biotypes described in this research, this reported biotype showed low resistance to the ALS inhibitor mesosulfuron-methyl with an RI of 2.6. Furthermore, this biotype showed high resistance to the ACCase inhibitor fenoxaprop-P-ethyl, with an RI value of 31.2 (Li et al., 2017).

Target-Site Resistance Mechanisms in This Biotype

Mutations involving the target gene *ALS* and *ACCase* is a common mechanism for ALS and ACCase inhibitor herbicide resistance. In this research, the *ALS* gene in the R biotype was shown to have a Pro-197-Ser mutation both by the SNPs and PCR analyses, this indicate the mutation may be one of a number of resistance mechanisms. Evolved resistance in weeds has been attributed to substitutions at each of the following eight different amino acids: Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 (Murphy and Tranel, 2019). Mutation at the site of 197 of the *ALS* gene was the most frequently reported mutation type in weed species, and was first found in *Kochia scoparia* (L.) Schrad (Saari et al., 1990). For *B. syzigachne*, only one kind of mutation (Pro-197-Ser) was found among the biotypes resistant to ALS inhibitor (Li et al., 2015). To date, seven kinds of amino acid substitutions in the CT domain of ACCase have been reported as being related to resistance to ACCase inhibitor herbicides in grassy weeds, and six substitutions have been found in *B. syzigachne*. In this research, no mutations were found in the R biotype, this indicated NTSR may be evolved in this biotype.

Common Metabolic Genes Relative to Multiple Resistance

Herbicide metabolism in weeds is a complex process involving metabolic enzymes (P450, GST, esterase, transporter, oxidase) and regulatory genes (transcription factors, protein kinases, and micro-RNAs) (Kreuz et al., 1996; Van Eerd et al., 2003; Yuan et al., 2007). *Beckmannia syzigachne* has evolved resistance to ACCase inhibitors, and many genes involved in NTSR were discovered using the RNA-seq technology (Pan et al., 2016a). Metabolic genes including CytP450, GST, esterases, peroxidases, ABC transporter B family members, and UDP-glycosyltransferases were identified (Pan et al., 2016a) Esterases break down herbicide molecules, and ABC transporters enhance resistance to herbicides by transporting isolated herbicides and acting as metabolites (Délye, 2013). In this research, one gene was annotated as an ABC transporter G family member, and one gene was annotated as UDP-glycosyltransferase 88F5 may be associated with resistance. Furthermore, two genes (*GST-T3* and *GST-U6*) were found to be overexpressed at different times after mesosulfuron-methly and fenoxaprop-p-ethyl treatment in resistant *B. syzigachne*. What is more, the GST activity in resistant plants higher than the susceptible ones at all the detected times. This result is similar to that reported in a

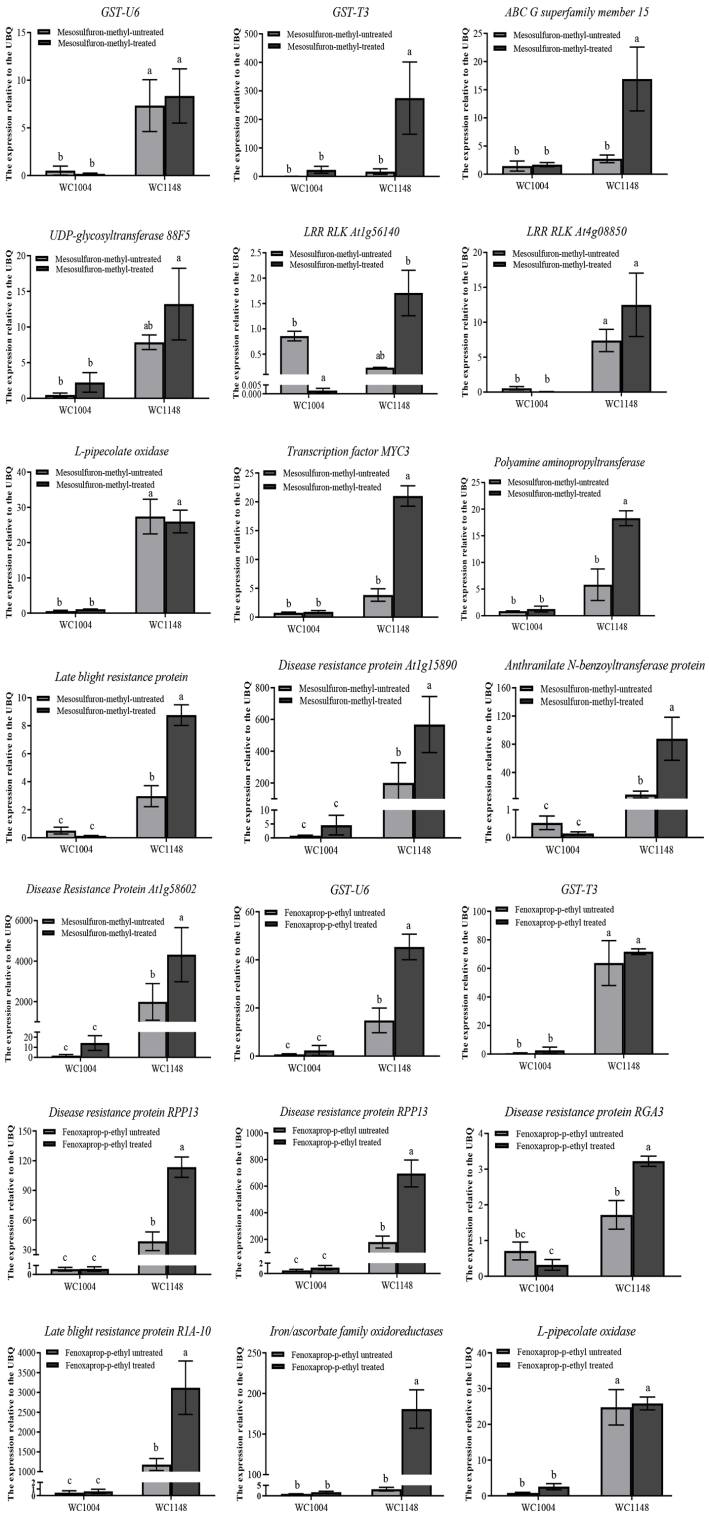

FIGURE 3 | Relative expression validations for candidate resistance genes using the qPCR method. The confirmed reference gene *UBQ* was selected as an internal control gene. The resistant biotype named WC1148 and the susceptible biotype named WC1004. Three biological replicates were performed for each treatment, and three technical replicates was done for each sample. Vertical bars show the standard error of the mean for the replicates. Different lowercase characters indicate significant differences in expression between the treatments.

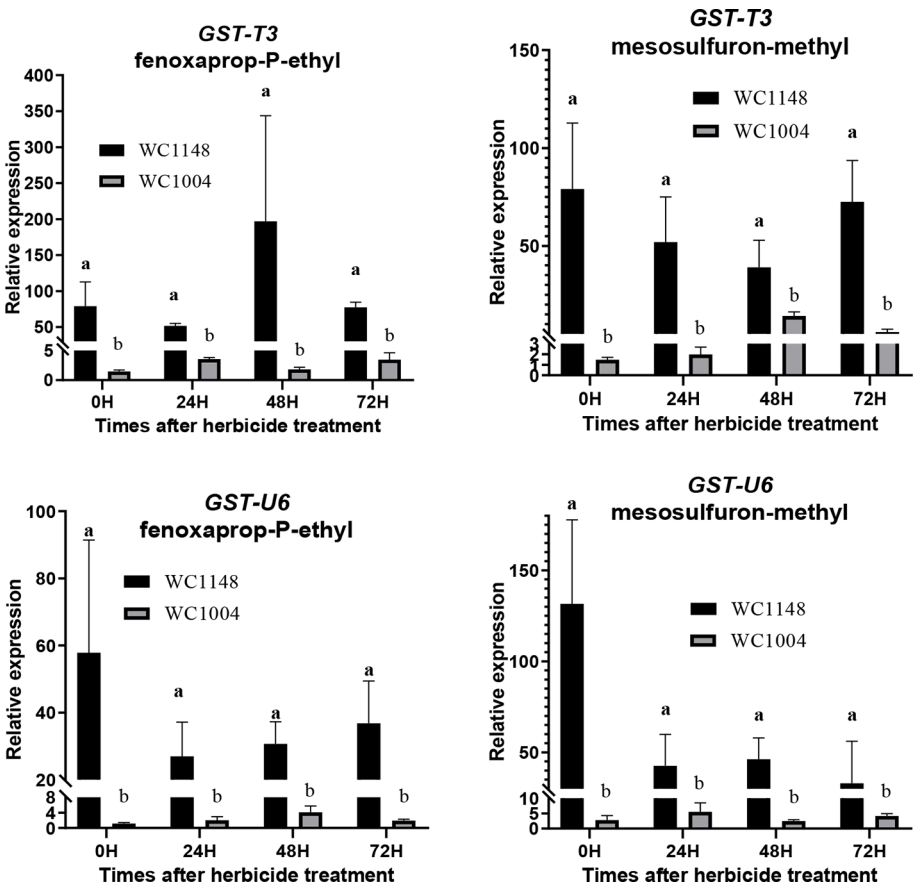

FIGURE 4 | The expression of two unigenes (*GST-T3*, *GST-U6*) annotation to GSTs in the resistant (R) and susceptible (S) plants at 0, 24, 48, and 72 h after mesosulfuron-methyl (20 g a.i. ha^{-1}) and fenoxaprop-P-ethyl (70 g a.i. ha^{-1}) treatment, respectively. At least three biological replicates were performed for each treatment, and three technical replicates was done for each sample. Bars are means ± standard error of the mean (SEM). Different lowercase means the significant difference at each time point between R and S which analyzed by the T-test ($P < 0.05$).

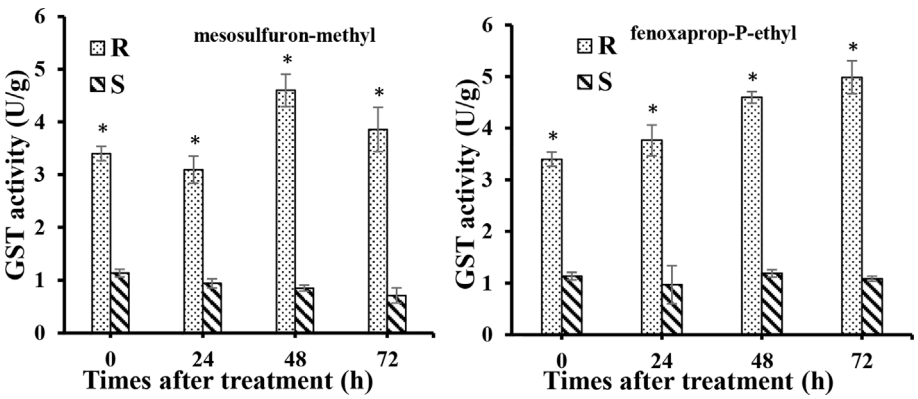

FIGURE 5 | GST activities in the resistant (R) and susceptible (S) plants at 0, 24, 48, and 72 h after fenoxaprop-P-ethyl (70 g a.i. ha^{-1}) and mesosulfuron-methyl (20 g a.i. ha^{-1}) treatment, respectively. At least three biological replicates were performed for each treatment. Bars are means ± standard error of the mean (SEM). *Significant difference at each time point between R and S ($P < 0.05$).

previous study (Pan et al., 2016b). This confirmed that these two genes are related to multiple resistance in this weed.

Novel Genes Related to Multiple Resistance

In addition to the reported resistance factors in *B. syzigachne*, some new genes related to resistance were also found in this research. Four genes annotated as encoding disease resistance proteins (one for *At1g15890*, one for *At1g58602*, and two for *RPP13*) in the resistant population were expressed at 65.8-1124.0 times higher levels than that in the sensitive biotype. This phenomenon was found both before and after herbicide treatment. This suggested that the disease-resistance protein might be related to the resistance. The expression levels of oxidase (L-pipecolate oxidase and iron/ascorbate family oxidoreductases) in R biotypes were 10.0–103.4 times higher than those in sensitive biotypes after treatment, which may be related to fenoxaprop-p-ethyl metabolism in individuals.

In this study, besides disease-resistant proteins, transcription factors and other regulatory genes were also screened. Protein kinases play key roles in signal transduction pathways, bringing external signals into cells, amplifying cascades of reactions, and passing them to transcription factor proteins to regulate the expression of downstream functional genes. Plant immune receptor is often classified as threonine kinase, and studies have shown that tyrosine phosphorylation in plants has an important regulatory role in innate immunity (Liu et al., 2018). MYC2 has a regulatory role in plant antioxidant capacity (Dombrecht et al., 2007). In this study, MYC3 transcription factors and protein kinase levels in WC11-48 expression were 22.8-917.2 times higher in the resistant biotype than that in the sensitive biotype, but the present study has not reported anything about the relationship between resistance genes and weeds. The roles of these genes in weed resistance mechanisms still need further research.

CONCLUSION

In this research, we found a *B. syzigachne* biotype that had evolved multiple resistance to ALS and ACCase inhibitor herbicides, and which showed high resistance to ALS inhibitor and relatively lower resistance to the ACCase ones. A Pro-197-Ser mutation was found in the *ALS* gene and no mutations were found in the *ACCase* gene. Four genes encoding disease resistance proteins, the MYC3 transcription factor, and one gene for blight resistance protein were found related to confer multiple resistance. Of the novel resistance factors, two GST genes (*GST-T3* and *GST-U6*) showed high correlation to this multiple resistance. The results of this research could provide the important resistant factors about multiple resistance to ACCase and ALS-inhibiting herbicide in *B. syzigachne*.

DATA AVAILABILITY STATEMENT

The raw Illumina sequence reads have been deposited in the NCBI Sequence Read Archive (SRA) database. The accession numbers for the samples with herbicide treatment were SRR10737896 and SRR10737895. And the accession numbers for the samples without herbicide treatment were SRR10742660 and SRR10742659.

AUTHOR CONTRIBUTIONS

This research was designed by HC. JW and JC performed the experimental work and the data analysis. XL, HC, DL, ZL, and JC provided helpful suggestions for the data analysis and manuscript revision. JC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.572610/full#supplementary-material

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Accumulation of Target Gene Mutations Confers Multiple Resistance to ALS, ACCase, and EPSPS Inhibitors in *Lolium* Species in Chile

José G. Vázquez-García[1], Ricardo Alcántara-de la Cruz[2], Candelario Palma-Bautista[1], Antonia M. Rojano-Delgado[1], Hugo E. Cruz-Hipólito[1], Joel Torra[3], Francisco Barro[4] and Rafael De Prado[1]*

[1] Department of Agricultural Chemistry and Edaphology, University of Córdoba, Córdoba, Spain, [2] Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil, [3] Department d'Hortofruticultura, Botànica i Jardineria, Agrotecnio, Universitat de Lleida, Lleida, Spain, [4] Department of Plant Breeding, Institute for Sustainable Agriculture, CSIC (IAS-CSIC), Córdoba, Spain

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***Correspondence:**
Francisco Barro
fbarro@ias.csic.es

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Different *Lolium* species, common weeds in cereal fields and fruit orchards in Chile, were reported showing isolated resistance to the acetyl CoA carboxylase (ACCase), acetolactate synthase (ALS) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibiting herbicides in the late 1990s. The first case of multiple resistance to these herbicides was *Lolium multiflorum* found in spring barley in 2007. We hypothesized that other *Lolium* species may have evolved multiple resistance. In this study, we characterized the multiple resistance to glyphosate, diclofop-methyl and iodosulfuron-methyl-sodium in *Lolium rigidum*, *Lolium perenne* and *Lolium multiflorum* resistant (R) populations from Chile collected in cereal fields. *Lolium* spp. populations were confirmed by AFLP analysis to be *L. rigidum*, *L. perenne* and *L. multiflorum*. Dose-response assays confirmed multiple resistance to glyphosate, diclofop-methyl and iodosulfuron methyl-sodium in the three species. Enzyme activity assays (ACCase, ALS and EPSPS) suggested that the multiple resistance of the three *Lolium* spp. was caused by target site mechanisms, except the resistance to iodosulfuron in the R *L. perenne* population. The target site genes sequencing revealed that the R *L. multiflorum* population presented the Pro-106-Ser/Ala (EPSPS), Ile-2041-Asn++Asp-2078-Gly (ACCase), and Trp-574-Leu (ALS) mutations; and the R *L. rigidum* population had the Pro-106-Ser (EPSPS), Ile-1781-Leu+Asp-2078-Gly (ACCase) and Pro-197-Ser/Gln+Trp-574-Leu (ALS) mutations. Alternatively, the R *L. perenne* population showed only the Asp-2078-Gly (ACCase) mutation, while glyphosate resistance could be due to *EPSPS* gene amplification (no mutations but high basal enzyme activity), whereas iodosulfuron resistance presumably could involve non-target site resistance (NTSR) mechanisms. These results support that the accumulation of target site

mutations confers multiple resistance to the ACCase, ALS and EPSPS inhibitors in *L. multiflorum* and *L. rigidum* from Chile, while in *L. perenne*, both target and NTSR could be present. Multiple resistance to three herbicide groups in three different species of the genus *Lolium* in South America represents a significant management challenge.

Keywords: glyphosate, diclofop-methyl, iodosulfuron methyl-sodium, italian ryegrass, perennial ryegrass, rigid ryegrass

INTRODUCTION

Lolium grasses are problematic weeds around the world (Salas et al., 2012; Menegat et al., 2016), causing yield losses in small grains, orchards, and vineyards, as well as in non-agricultural lands (Travlos et al., 2018). There are 17 *Lolium* species described, but the most problematic ones in agricultural producing systems are *L. multiflorum* (Lam.) Husnot (Italian ryegrass), *L. perenne* L. (perennial ryegrass) and *L. rigidum* Gaudin (rigid ryegrass) (Menegat et al., 2016). The last species is considered the most economically important weed in Australia, while *L. multiflorum* and *L. perenne* are frequents weeds in crops of North and South America and the Mediterranean region (Heap, 2020).

Over the past three decades, control of *Lolium* grasses has been carried out using almost exclusively PRE- and POST-emergence herbicides, exerting high selection pressure that has resulted in resistance to most herbicide sites of action (SoAs) (Tehranchian et al., 2018). The first case of resistance in *L. rigidum* was found in canola, spring barley, and wheat fields in Australia in 1982. Since then, herbicide resistance cases in *Lolium* species have increased substantially over time (Heap, 2020). There are currently 67, 9, and 48 cases of herbicide resistance in *L. multiflorum*, *L. perenne*, and *L. rigidum*, respectively, recorded in the database of the *International Survey of Herbicide Resistant Weeds*. At least half of these are cases of cross and/or multiple resistance (Heap, 2020).

Lolium grasses are difficult to control chemically because they have the capacity to evolve different herbicide resistance mechanisms very quickly, depending on the distribution of resistant individuals within the cultivated areas, seed viability, obligated cross-pollination, genetic variability and high phenotypic plasticity (Travlos et al., 2018). For example, *L. rigidum* has evolved resistance to 14 different herbicide SoAs, and *L. multiflorum* has evolved resistant to 9 SoAs (Heap, 2020). In addition, because *Lolium* weed species generally are diploids (Dalton et al., 1999; Busi and Powles, 2013), they are capable of hybridizing with each other, resulting in populations with homozygous and heterozygous individuals that can carry multiple resistance alleles (Menegat et al., 2016). In the long term, this situation reduces the options for diversification of alternative herbicides for the integrated management of these weeds (Fernández et al., 2017). In addition, resistance alleles are generally not linked and accumulate independently, i.e., *Lolium* species are able to accumulate different resistance mechanisms, so different combinations of resistance alleles can be found resulting in complex multiple herbicide resistance profiles (Tan et al., 2007; Martins et al., 2014). The most serious multiple herbicide resistance cases are those including resistance to glyphosate (5-enolpyruvylshikimate-3-phosphate synthase [EPSPS] inhibitor) (Fernández-Moreno et al., 2017a). Populations of *L. multiflorum* showing resistance to glyphosate, sethoxydim (acetyl CoA carboxylase [ACCase] inhibitor), and paraquat have been found in the United States (Tehranchian et al., 2018); *L. rigidum* populations resistant to glyphosate, paraquat, ACCase and acetolactate synthase (ALS) inhibitors have been found in Australia (Yu et al., 2007, 2008). Additionally, different populations of *Lolium* species with multiple resistance to up to four herbicide SoAs have been reported throughout the Mediterranean region in Europe (Collavo and Sattin, 2014; Fernández et al., 2017; Travlos et al., 2018).

In Chile, herbicide resistance is not recent, and of the 19 known cases, nine involve *Lolium* species with single and/or multiple resistance occurring in small grain fields (barley, canola, cereals, lupins, spring and winter wheat) and fruit orchards (Heap, 2020). In Chile, *L. rigidum*, *L. multiflorum* and *L. perenne* were found with resistance to ACCase inhibitors in small grains fields in 1997, 1998 and 2001, respectively (Heap, 2020). *Lolium multiflorum* was the first species to show resistance to glyphosate, and it was found in fruit orchards in 2001 (Perez and Kogan, 2003). The first case of resistance to ALS inhibitors was also *L. multiflorum*, found in wheat fields in 2002, and this case was the first one of multiple resistance, since this species also showed resistance to glyphosate (Heap, 2020). Because ACCase- and ALS-inhibiting herbicides became recurring alternatives to manage glyphosate resistance and vice versa, we hypothesized that populations of *Lolium* species from Chile may have had multiple resistance to these three herbicide groups, since they can select herbicide resistance in few generations, even when exposed to subdoses (Busi et al., 2012). However, the resistance mechanisms were characterized only for *L. multiflorum* resistance to glyphosate (Perez and Kogan, 2003; Michitte et al., 2007). In addition, herbicide resistance in *Lolium* species is widely dispersed in neighboring Latin American countries (Heap, 2020), and only in Brazil is it estimated that *L. multiflorum* affects more than 4.2 million ha (Alcántara-de la Cruz et al., 2020). However, with the exception of the recent characterization of the resistance mechanisms to ACCase inhibitors in *L. perenne* from Argentina (Yanniccari and Gigón, 2020), there are no studies of resistance mechanisms in *Lolium* species from South America (*L. multiflorum* and *L. perenne*) other than for glyphosate (Yanniccari et al., 2017; Barroso et al., 2018).

In this work, the multiple resistances to glyphosate, diclofop-methyl and iodosulfuron-methyl-sodium (inhibiting herbicides of EPSPS, ACCase and ALS enzymes, respectively) were characterized in three putative resistant (R) populations of *L. rigidum*, *L. perenne* and *L. multiflorum*, collected in spring barley and winter wheat fields in the Regions VIII

(San Bernardo and Olivar) and IX (Vilcún) in Chile. The possible target-site resistance (TSR) mechanisms involved were studied in these three populations, being a one-of-a-kind study for South America.

MATERIALS AND METHODS

Chemicals

Trade formulations of glyphosate (Roundup 480, Monsanto Europe), diclofop-methyl (Firelo, Dupont Spain) and iodosulfuron-methyl-sodium (Hussar, Bayer CropScience Spain) were used for the dose-response assays. Glyphosate, diclofop-acid and iodosulfuron of analytical grade (Sigma-Aldrich, Spain) with a purity of 99% were used for enzymatic assays.

Plant Material

Seeds of the different R *Lolium* spp. (*L. rigidum*, *L. perenne*, and *L. multiflorum*) were supplied in 2010 by Dr. Nelson Espinoza (INIA of Carillanca, Chile). Mature seeds were collected from different fields of spring barley (Region VIII) and winter wheat (Region IX) grown under direct drilling systems for several years (Espinoza et al., 2009). Spring weeds were removed in pre-sowing with glyphosate, and during the crop season, ALS- (iodosulfuron) and/or ACCase- (diclofop, haloxyfop, and others) inhibiting herbicides were used for weed control. A screening test was conducted on the R populations to eliminate susceptible individuals from the field-collected seed, which consisted of germinating the seeds of the putative R populations and treating the seedlings at field doses with diclofop-methyl (900 g ai ha^{-1}), glyphosate (720 g ae ha^{-1}) and iodosulfuron (5 g ai ha^{-1}). Individuals surviving these applications were allowed to grow to maturity to collect the purified seeds used in this study. Seeds of populations of each *Lolium* species that were susceptible (S) to these herbicides were collected in areas near these crops.

Seeds of the R and S *Lolium* spp. populations were placed for germination in 9-cm Petri dishes containing moistened filter paper. Petri dishes were placed in a growth chamber at 26/18°C (day/night), with relative humidity of 60% and a photoperiod of 16 h at a light density of 850 mmol m^{-2} s^{-1}. Seedlings were transplanted into plastic pots in 250 mL substrate (sand and peat 1:1). Pots were returned to the growth chamber, and the plants were irrigated as necessary until ready for use (plants having 3–4 true leaves).

Molecular Characterisation of *Lolium* Species

One population of each *Lolium* species, i.e., *L. multiflorum*, *L. rigidum* (harvested in southern Spain) and *L. perenne* (North of Portugal) were included as reference populations in the molecular analysis with amplified fragment length polymorphism (AFLP) markers. The molecular identity of the reference populations has been previously confirmed. Twelve plants of each *Lolium* spp. population were used for DNA extraction from leaf tissue (50 mg $plant^{-1}$) by using the Speedtool DNA Extraction Plant kit (Biotools, Madrid, Spain). DNA was quantified in a NanoDrop ND-1000 spectrophotometer. AFLP analysis was carried out using the fluorescent AFLP IRDye kit for Large Plant Genome Analysis (LI-COR Biosciences) following the manufacturer's instructions. Twelve primer pairs [E36-M48 (E-ACC MCAC)/E36-M60 (E-ACC MCTC)/E37-M49 (E-ACG MCAG)/E38-M50 (E-ACT MCAT)/E40-M61 (E-AGC MCTG)/E35-M49 (E-ACA MCAG)/E36-M49 (E-ACC MCAG)/E35-M61 (E-ACA MCTG)/E40-M62 (E-AGC MCTT)/E32-M60 (E-AAC MCTC)/E33-M50 (E-AAG MCAT)/E35-M48 (E-ACA MCAC)] were used for selective amplification (Fernández et al., 2017; Fernández-Moreno et al., 2017a,b). AFLP products were separated in a polyacrylamide electrophoresis using an automated sequencer (LICOR 4300). Polymorphic AFLP markers (12) and primers were identified, and individuals were scored for presence (1) or absence (0) of AFLP fragments using the software package SAGAMX 2 GENERATION. Genetic distances were calculated using Jaccard's coefficients of similarity. Grouping of the genotypes was determined by using the unweighted pair group method with arithmetic mean (UPGMA). The analysis was performed with AFLP marker data in the program NTSYSpc 2.2.

Dose–Response Assays

R and S *Lolium* spp. plants were treated with different doses of diclofop (0, 100, 200, 400, 800, 1,600, 3,200, 4,000 g ai ha^{-1}), glyphosate (0, 31.25, 62.5, 125, 250, 500, 1,000, 1,500, 2,000, 4,000 g ae ha^{-1}) and iodosulfuron (0, 1, 2, 4, 8, 16, 32, 64, 128 g ai ha^{-1}) in a laboratory chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, United States) equipped with 8002 flat fan nozzles and delivering 200 L ha^{-1} at 250 KPa. The experiments were based on a completely random design with eight replications by dose; and experiments were repeated. Plant mortality and above-ground fresh weight were evaluated 28 days after treatment (DAT), and data were expressed as the percentage of the untreated control. Herbicide rates reducing the plant growth (GR_{50}) or causing plant mortality by 50% (LD_{50}) with respect to the untreated control were determined for each *Lolium* spp. population and herbicide.

Enzyme Activity Assays

EPSPS Activity

The extraction and activity of the EPSPS enzyme was carried out following the methodology described by Dayan et al. (2015). Five grams of foliar tissue from the different *Lolium* spp. populations were ground with liquid N_2 to a fine powder. Samples were transferred to 50 mL Falcon tubes containing 25 mL of extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine) with 70 μL of ß-mercaptoethanol (10 mM) and 1% in polyvinylpolypyrrolidone (PVPP) to extract the EPSPS. Samples were vortexed for 5 min, avoiding foaming, and then centrifuged (18,000 *g*, 30 min, 4°C). Supernatant was filtered using a cheesecloth in a cold beaker and then slowly added $(NH_4)_2SO_4$, was then slowly added while supernatant was under continuous stirring until a 45% solution of $(NH_4)_2SO_4$ (w/v) was obtained. After addition of $(NH_4)_2SO_4$ the sample was stirred for 30 min and then centrifuged (15,000 *g*, 30 min, 4°C). This step was repeated once

more to obtain a 70% $(NH_4)_2SO_4$ (w/v) solution to precipitate the fraction that contained the EPSPS activity. Supernatants were discarded, and pellets were resuspended in 1–2 mL assay buffer (100 mM MOPS, 1 mM $MgCl_2$, 10% glycerol (v/v), 2 mM sodium molybdate, 200 mM NaF). Samples were dialysed using Slide-A-Lyzer dialysis cassettes (1000-MWC, Thermo Scientific, Meridian, IL, United States) overnight in 2 L of dialysis buffer (100 mM MOPS and 5 mM EDTA) at 4°C on a stir plate. The final pH of the buffers was adjusted to 7.0 with HCl or NaOH. The concentration of total protein soluble (TPS) was determined by Bradford assay (Bradford, 1976). Basal and enzyme EPSPS activities were determined in a continuous assay quantifying the inorganic phosphate (Pi) released from shikimate-3-phosphate with the EnzCheck phosphate assay kit (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. The glyphosate concentrations used were: 0, 0.1, 1, 10, 100 and 1,000 μM. The amount Pi released was measured for 10 min at 360 nm in a spectrophotometer (DU-640, Beckman Coulter Inc. Fullerton, CA, United States). The EPSPS activity was calculated by determining the amount of Pi (μmol) released in μg of TSP^{-1} min^{-1}. EPSPS enzyme activity was expressed as percentage of enzyme activity in presence of glyphosate with respect to the control (basal activity without glyphosate). The experiment was repeated for all populations, and each glyphosate concentration had three technical replicates.

ACCase Activity

The *in vitro* ACCase enzyme activity was performed following the protocol described by De Prado et al. (2005). Six grams of fresh weight were taken from new leaves of the different *Lolium* spp. populations for the extraction step. The samples were ground using liquid N_2 and then added to 24 mL extraction buffer (0.1 M Hepes-KOH at pH 7.5, 0.5 M glycerol, 2 mM EDTA and 0.32 mM PMSF). After mixing for 3 min with a magnetic stirrer and then filtered using a cheesecloth, the samples were centrifuged (24,000 *g*, 30 min, 4°C). the supernatant was fractioned with $(NH_4)_2SO_4$ and centrifuged (12,000 *g*, 10 min at 4°C). The pellets were resuspended in 1 mL S400 buffer (0.1 M Tricine-KOH at pH 8.3, 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA and 0.5 mM DTT). The homogenate was applied to a desalting column (PD-10 columns, Sephadex G-25 M, Amersham Biosciences AB, SE-751 84, Uppsala, Sweden) and eluted in 2 mL of S400 buffer. The specific protein concentrations were determined by Bradford assay (Bradford, 1976). The enzyme activity was measured through the ATP-dependent incorporation of $NaH[^{14}C]O_3$ into [^{14}C]malonyl-CoA. The reaction was conducted at 34°C in 7 mL scintillation vials with 0.1 M Tricine-KOH at pH 8.3, 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, 0.5 mM DTT, 1.5 mM $MgCl_2$, 15 mM $NaH[^{14}C]O_3$ (1.22 MBq μmol^{-1}), 50 μL of enzyme extract, 5 mM acetyl-CoA in a final volume of 0.2 mL. The reaction was stopped after 5 min by adding 30 μL of HCl 4N. After the drying step, 0.5 mL of ethanol-water solution (1:1, v/v) was added to the vial, followed by 5 mL of scintillation cocktail (Ultima Gold, Perkin-Elmer, BV BioScience Packard). Radioactivity was determined by liquid scintillation spectrometry. One unit of ACCase activity was defined as 1 μmol malonyl CoA formed min^{-1}. The diclofop-acid concentrations were: 0, 0.1, 1, 10, 100, 1,000 and 10,000 μM. The experiment was repeated with three replicates for each herbicide concentration.

ALS Activity

ALS activity in presence of different iodosulfuron concentrations was determined *in vitro* following the protocol described by Rojano-Delgado et al. (2015). Three grams of young leaf tissue were powdered using liquid N_2. Polyvinylpyrrolidone (0.5 g) was added to the fine powder as well as extraction buffer [1M K-phosphate buffer solution (pH 7.5), 10 mM sodium pyruvate, 5 mM $MgCl_2$, 50 mM thiamine pyrophosphate, 100 μM flavin adenine dinucleotide, 12 mM dithiothreitol and glycerol–water (1:9, v/v)] in a proportion of 1:2 tissue–buffer. Samples were agitated for 10 min at 4°C, filtered through a cheesecloth, and centrifuged (20,000 *g* for 20 min). The supernatant was immediately used for the enzyme assays. For the ALS enzyme activity, 90 μL of enzyme extract was used with 110 μL of freshly prepared assay buffer [0.08 M K-phosphate buffer solution (pH 7.5), 0.5 M sodium pyruvate, 0.1 M $MgCl_2$, 0.5 mM thiamine pyrophosphate and 1,000 μM flavin adenine dinucleotide]. The iodosulfuron concentrations assayed were: 0, 0.1, 1, 10, 50, 100, 500, 1,000 and 5,000 μM. Aliquots of 250 μL of a solution 0.04 M K_2HPO_4 at pH 7.0 were added. This mixture was incubated for 1 h at 37°C. Afterward, the reaction was stopped by adding 50 μL of H_2SO_4/water 1:10 (v/v). To decarboxylate acetolactate to acetoin, the tubes with the mixture were heated for 15 min at 60°C. A colored complex (λ 520 nm) formed after the addition of 250 μL of creatine (5 g L^{-1} freshly prepared in water) and 250 μL of 1-naphthol (50 g L^{-1} freshly prepared in 5 N NaOH) prior to incubation at 60°C for 15 min. Total protein content was determined by the Bradford method (Bradford, 1976), based on measurement of the absorbance at 595 nm of an acidic solution of Coomassie Brilliant Blue G-250 after binding to proteins. The experiment was repeated and with three replicates for each concentration of the herbicide.

RNA Extraction and Target-Site Genes Sequencing

Samples (∼100 mg) of foliar tissue were taken from 20 seedlings from the R and S *Lolium* spp. populations. Then, both R plants were treated with glyphosate, diclofop and iodosulfuron at a rate of 720 (g ae ha^{-1}), 400 and 5 g ai ha^{-1}, respectively, as was done in the dose-response assays. Only R plants that survived these application rates 21 DAT were considered for molecular analyses (Cruz-Hipolito et al., 2015). Total RNA was isolated from leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. RNA was treated with TURBO DNase (RNase-Free; Ambion, Warrington, United Kingdom) to eliminate any DNA contamination and stored at −80°C. First strand complementary DNA (cDNA) synthesis was carried out with 2 μg RNA per sample using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, United States). The primers to amplify the ACCase (two fragments), ALS (two fragments), EPSPS genes and the PCR conditions are described in **Table 1**. Each PCR reaction was conducted in a total volume of 25 μL [50 ng of cDNA, 0.2 μM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems;

TABLE 1 | Primers and PCR conditions used to amplify the 5-enolpiruvylshikimate-3-phosphate synthase (*EPSPS*), acetyl-coenzyme A carboxylase (*ACCase*, two fragments) and acetolactate synthase (*ALS*) genes to identify potential mutations responsible for herbicide resistance in *Lolium* species in Chile.

Target gene	Primers	Sequences 5′→3′	Fragment length	References	PCR conditions
EPSPS	Forward	AGCTGTAGTCGTTGGCTGTG	120 bp	Fernández-Moreno et al., 2017b	1 cycle at 94°C for 3 min; 35 cycles of 94°C for 30 s; 55°C for 30 s and 72°C for 60 s; and a final extension step of 10 min at 72°C.
	Reverse	GCCAAGAAATAGCTCGCACT			
ACCase	CP1-F	CAACTCTGGTGCTIGGATIGGCA	551 bp	Martins et al., 2014	1 cycle at 95°C for 30 s; 37 cycles of 95°C for 10 s; 60°C for 15 s and 72°C for 45 s; and a final extension step of 10 min at 72°C.
	CP1-R	GAACATAICTGAGCCACCTIAATATATT			
	ACCF2	ATCCTCGTGCAGCCATAAGTG	510 bp	Tehranchian et al., 2018	1 cycle at 95°C for 3 min; 40 cycles of 95°C for 30 s; 57°C for 45 s and 72°C for 60 s; and a final extension step of 5 min at 72°C.
	ACCR2	TGCATTCTTGGAGTTCCTCTG			
ALS	ALSF197	ACTCCATCCCCATGGTGGC	1,449 bp	Yu et al., 2008	1 cycle at 94°C for 4 min; 35 cycles of 94°C for 30 s; 55°C for 30 s and 72°C for 90 s; and a final extension step of 5 min at 72°C.
	ALSR653	TCCTGCCATCACCTTCCATG			

Life Technologies S.A., Spain), 2 mM MgCl2, 1X buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain)]. Each cDNA sample, i.e., cDNA of each plant, was amplified in triplicate. An aliquot of the PCR product (10 μL) was loaded in a 1% agarose gel to check the correct band amplification. The rest of the PCR product (15 μL) was purified using ExoSAP-IT® for PCR Product Clean-Up (USB, Cleveland, OH, United States) as indicated by the manufacturers and sequenced. Sanger sequencing was carried out by the SCAI DNA sequencing service of the University of Córdoba, Spain. Gene sequences of the R and S *Lolium* species were verified and assembled using the Geneious 8.1.8 (Biomatters Ltd, Auckland, New Zealand) software, to identify mutations responsible for conferring herbicide resistance.

Statistical Analysis

Dose-response and enzyme activity data were subjected to non-linear regression analysis using the log-logistic equation $Y = d/1+(x/g)^b)$; where Y represents the percentage of fresh weight reduction, plant survival and enzyme activity inhibition with respect to the control, *d is* the limits of the upper asymptote, b is the slope of the curve, g is the herbicide rate at the inflection point (i.e., GR_{50}, LD_{50} or I_{50}), and x (independent variable) is the herbicide dose. Using this equation, the GR_{50}, LD_{50} and I_{50} (herbicide concentration to inhibit the ACCase, EPSPS and/or ALS activity by 50%) of each population of each species were calculated. Regression analyses were conducted using the '*drc*' package with program R version 3.2.5 (Ritz et al., 2015). Resistance factors (RF = R/S) were calculated as the ratio of R to-S GR_{50}, LD_{50} or I_{50}.

Specific enzyme activity data were submitted to one-way ANOVA (for $P < 0.05$). The Tukey test was used for mean comparison (95% confidence level) to test for significant differences between populations within each species and for each enzyme.

RESULTS

Molecular Characterisation of *Lolium* Species

The UPGMA analysis clustered the *Lolium* spp. populations into a large group and two subgroups. In the larger group, the Jaccard index indicated a 62% similarity between *L. rigidum*, *L. perenne*, and *L. multiflorum*. In the first subgroup, the putative R and S *L. rigidum* populations from Chile were confirmed to belong to this species, because they were grouped together to the reference population from Spain. In the second subgroup, *L. multiflorum* and *L. perenne* populations were found to have 80% similarity. The putative R and S populations from Chile of each of these two *Lolium* species were grouped with their respective reference population from Portugal (*L. perenne*) and Spain (*L. multiflorum*), confirming their identities at molecular level (**Figure 1**).

Glyphosate Dose–Response Assays

The fresh weight of the S *Lolium* spp. populations was reduced by 50% with glyphosate doses close to 100 g ae ha^{-1} (**Figures 2A,B**). The R populations of *L. perenne*, *L. rigidum*, and *L. multiflorum* were 5- (GR_{50} = 453 g ae ha^{-1}), 6.6- (GR_{50} = 689 g ae ha^{-1}) and 12.5- (GR_{50} = 937 g ae ha^{-1}) times more resistant to glyphosate, respectively, in relation to their respective S counterparts. Regarding survival (LD_{50}), the RF values of the R populations of *L. perenne*, *L. rigidum* and *L. multiflorum* were 5.6, 7.1, and 14.1, respectively. These RF values were determined according to the LD_{50} (210, 258 and 290 g ae ha^{-1} glyphosate) values of the S populations following the same species order (**Table 2**).

Diclofop Dose–Response Assays

Diclofop resistance of the R *Lolium* spp. populations followed the same trend as glyphosate resistance, i.e., the R population of *L. perenne* had the lowest resistance level, followed by *L. multiflorum* (intermediate resistance level), while *L. rigidum* was the most resistant species, both in relation to the percentage of fresh weight reduction as well the plant survival rate by 50% (**Figures 2C,D**). The GR_{50} values of the S populations ranged from 148 to 254 g ai ha^{-1} diclofop, and the LD_{50} values were between 269 and 388 g ai ha^{-1}. The RF of the R populations ranged from 6.4 to 11.4 and from 4.4 to 9.4 in relation to the GR_{50} and LD_{50} values, respectively, of the S populations (**Table 3**).

Iodosulfuron Dose–Response Assays

The R populations of the three *Lolium* species survived iodosulfuron rates higher than the recommended field dose (3.5–5 g ai ha^{-1}) (**Figures 2E,F**). Based on weight reduction,

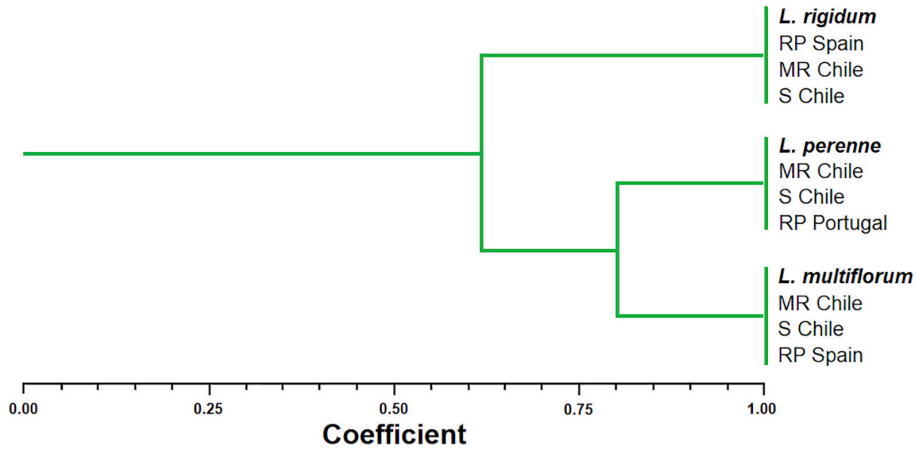

FIGURE 1 | Dendrogram of the genetic similarities among multiple herbicide resistant (MR) and susceptible (S) populations of *Lolium* species from Chile in comparison to reference's populations (RP) from Portugal and Spain after the UPGMA analysis performed with AFLP marker data. Twelve plants of each putative population were used for the molecular analysis.

the least resistant species was *L. perenne* (GR_{50} = 27.0 g ai ha^{-1}), followed by *L. multiflorum* (GR_{50} = 50.2 g ai ha^{-1}). *Lolium rigidum* (GR_{50} = 66.8 g ai ha^{-1}) was the most resistant species. The R populations were between 9 and 24 times more resistant to iodosulfuron than the S populations. These levels of resistance decreased in relation to the survival rate, where the LD_{50} values of the S populations ranged from 4.9 to 6.2 g ai ha^{-1} and those of R populations from 36 to 82.8 g ai ha^{-1}. The RF of the R populations in relation to the S ones were 7.3, 11.2, and 13.4 for *L. perenne*, *L. multiflorum*, and *L. rigidum*, respectively (**Table 4**).

Enzyme Activity Assays

The specific activity of the EPSPS was similar between R and S populations of *L. multiflorum* (0.037 μmol Pi μg^{-1} TSP min^{-1}) and *L. rigidum* (0.026 μmol Pi μg^{-1} TSP min^{-1}); however, differences of such activity were observed between *L. perenne* populations [0.032 (S) vs 0.133 (R) μmol Pi μg^{-1} TSP min^{-1}]. The specific ACCase activity, that ranged from 10.3 to 17.2 nmol HCO_3^- mg $protein^{-1}$ min^{-1}, was lower in the R populations of the three *Lolium* species. In the case of the specific ALS activity, there were differences between species, but not among R and S populations within each *Lolium* species. The averages were 1,511, 1,752, and 1,293 nmol acetoin mg^{-1} protein h^{-1} for *L. multiflorum*, *L. perenne*, and *L. rigidum*, respectively (**Figures 3A,C,E**).

The R populations of *L. multiflorum*, *L. perenne*, and *L. rigidum* required 14.2, 8.4, and 12.3 times, respectively, more glyphosate to inhibit EPSPS activity by 50%; and 27.7, 11.1, and 29.6 times more diclofop to inhibit ACCase in the same proportion in relation to their respective S counterparts. The ALS activities of the R populations of *L. multiflorum* and *L. rigidum* in response to iodosulfuron were 13.4 and 23.2 times lower, respectively, than the S populations; however, no differences in the sensitivity of this enzyme were observed between the R and S populations of *L. perenne* (**Figures 3B,D,F** and **Table 5**).

Target Site Changes

Nucleotide substitutions were found at the 106-CCA codon, which naturally encodes to proline, in the *EPSPS* gene, in the R *L. multiflorum* and *L. rigidum* populations (**Table 6**). The most frequent codon substitution was TCA (serine) found in 8 and 12 resistant individuals of *L. multiflorum* and *L. rigidum*, respectively. In addition, five R individuals of *L. multiflorum* presented the ACA (alanine) nucleotide combination. Regarding the *ACCase* gene, the three R populations of *Lolium* species showed different combinations of amino acid substitutions. Four and five R individuals of *L. multiflorum* presented the mutations Ile-2041-Asn and Asp-2078-Gly, respectively; *L. perenne* presented only the Asp-2078-Gly mutation (7 individuals); and *L. rigidum* presented the mutations Ile-1781-Leu and Asp-2078-Gly (5 and 8 individuals, respectively). Finally, the most frequent amino acid substitution found in the *ALS* gene of the R *L. multiflorum* and *L. rigidum* populations was Trp-574-Leu (11 and 6 individuals, respectively). In addition, two different amino acid substitutions were found at the Pro-197 position of the *ALS* gene in four (Ser) and three (Gln) individuals of the R *L. rigidum* population. No nucleotide changes were detected in the R *L. perenne* population (**Table 6**).

DISCUSSION

Differentiation between *Lolium* species on the basis of phenotypic characteristics is difficult (Menegat et al., 2016), therefore, a molecular characterisation was required to establish the molecular identities between the three populations studied. AFLP analyses make it possible to differentiate which populations belong to each *Lolium* species based on their molecular relationships (>90% similarity), by comparing them with their respective reference species of *L. multiflorum*, *L. perenne* and *L. rigidum*. However, this molecular tool did not allow differentiating the susceptibility or resistance status to herbicides

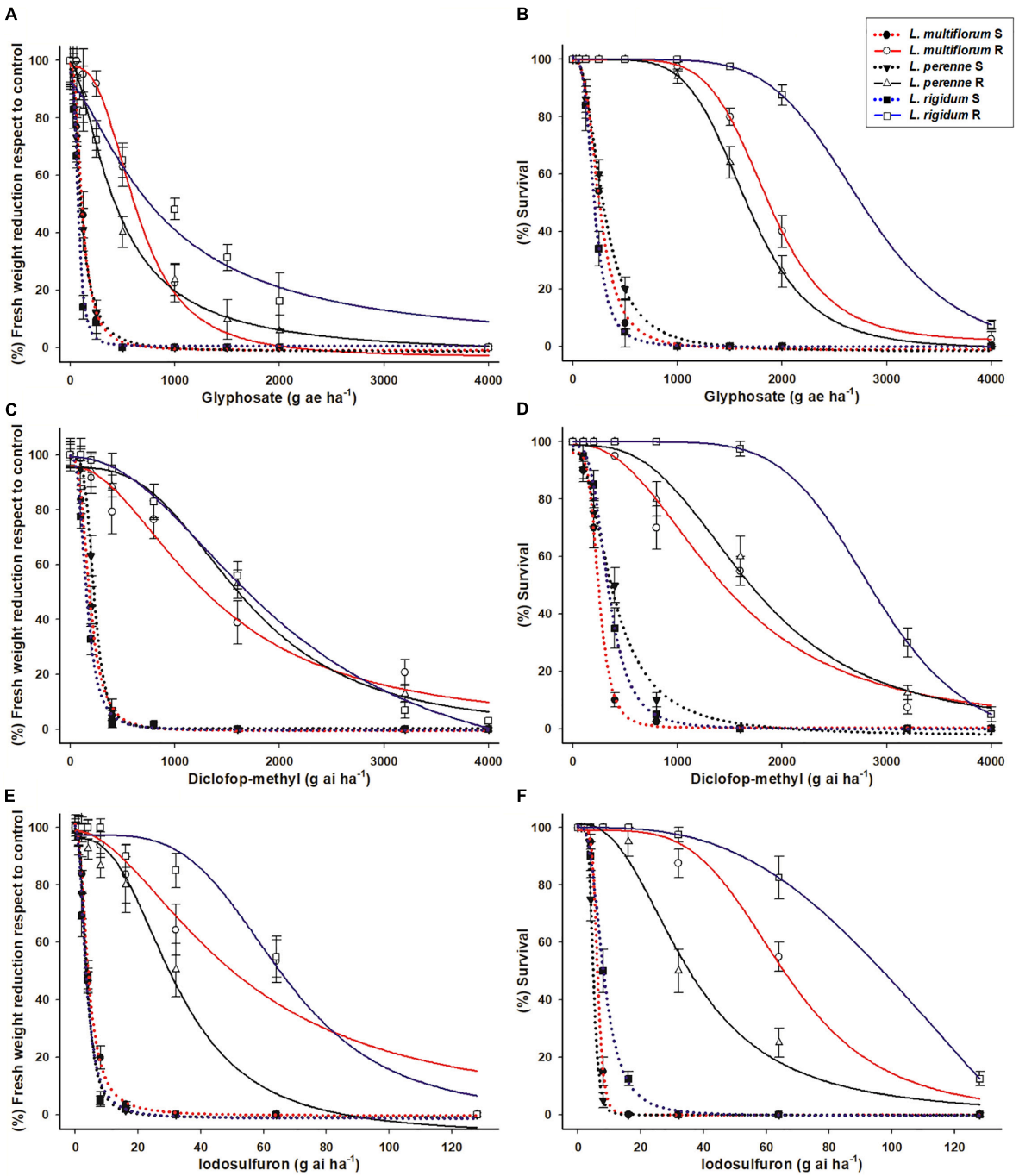

FIGURE 2 | Dose-response curves (fresh weight reduction and survival rates) of glyphosate **(A,B)**, diclofop-methyl **(C,D)** and iodosulfuron **(E,F)** in resistant (R) and susceptible (S) populations of *Lolium* species from Chile. Vertical bars ± standard error (n = 8).

since the genetic similarity between R and S populations within each species was equal to or greater than 95%. Due to the lack of homology and size homoplasy of fragments between individuals and populations, AFLP analyses may give biased results (Caballero et al., 2008; Fernández-Moreno et al., 2017a). For example, glyphosate R and S populations of *Amaranthus palmeri* presented high similarity levels of genetic variation (Chandi et al., 2013), as well as populations of *Spartina alterniflora* from different coastal regions of China that showed a weak genetic differentiation (Li et al., 2019). Therefore, AFLPs analyses are important to understand genetic diversity between and within weed populations, but these studies are not so crucial to optimize weed management programs (Sterling et al., 2004).

TABLE 2 | Parameters of the sigmoidal equation[a] used to estimate the effective mean dose of glyphosate (EPSPS inhibitor) required to reduce the fresh weight (GR_{50}) and plant survival (LD_{50}) by 50% in multiple-resistant (R) and -susceptible (S) populations of *Lolium* species in Chile.

Species	Population	d	b	R^2	Mean dose	RF	*P*-value
Growth reduction (GR_{50})							
L. multiflorum	R	98.8	2.2	0.99	689.2	6.6	<0.0001
	S	99.5	1.9	0.98	103.7		
L. perenne	R	102.4	1.4	0.99	452.9	5.0	<0.0001
	S	102.2	1.8	0.99	90.7		
L. rigidum	R	100.2	1.5	0.99	936.6	12.5	<0.0001
	S	100.9	1.8	0.99	74.9		
Plant survival (LD_{50})							
L. multiflorum	R	99.7	3.5	0.99	1,846.1	7.1	<0.0001
	S	102.4	1.8	0.99	258.3		
L. perenne	R	100.2	3.6	0.99	1,634.4	5.6	<0.0001
	S	102.6	1.8	0.99	290.0		
L. rigidum	R	100.5	2.0	0.99	2,960.1	14.1	<0.0001
	S	102.8	1.9	0.99	210.2		

[a] *Y = d/1+(x/g): where Y = percentage of fresh weight with respect to the untreated control, d = upper limit, b = slope of the curve, and g = herbicide concentration at the inflection point (i.e., GR_{50} or LD_{50}), and x = herbicide concentration. Resistance factors (RF = R/S) are the ratio of R to-S GR_{50} or LD_{50}. P is the level of significance of the non-linear model.*

TABLE 3 | Parameters of the sigmoidal equation[a] used to estimate the effective mean dose of diclofop-methyl (ACCase inhibitor) required to reduce the fresh weight (GR_{50}) and plant survival (LD_{50}) by 50% in multiple-resistant (R) and -susceptible (S) populations of *Lolium* species in Chile.

Species	Population	d	b	R^2	Mean dose	RF	*P*-value
Growth reduction (GR_{50})							
L. multiflorum	R	100.5	1.4	0.99	1370.9	7.6	0.0001
	S	97.2	1.3	0.98	179.3		
L. perenne	R	99.6	1.7	0.99	1643.7	6.4	0.0001
	S	100.5	1.1	0.99	254.9		
L. rigidum	R	99.9	1.7	0.99	1698.7	11.4	0.0001
	S	99.3	1.0	0.99	148.9		
Plant survival (LD_{50})							
L. multiflorum	R	100.0	1.6	0.99	1476.8	5.5	0.0001
	S	102.9	1.3	0.99	269.7		
L. perenne	R	99.8	1.9	0.99	1701.1	4.4	0.0001
	S	100.7	1.5	0.99	388.1		
L. rigidum	R	100.5	2.3	0.99	2896.9	9.4	0.0001
	S	101.5	1.6	0.99	309.1		

[a] *Y = d/1+(x/g): where Y = percentage of fresh weight with respect to the untreated control, d = upper limit, b = slope of the curve, and g = herbicide concentration at the inflection point (i.e., GR_{50} or LD_{50}), and x = herbicide concentration. Resistance factors (RF = R/S) are the ratio of R to-S GR_{50} or LD_{50}. P is the level of significance of the non-linear model.*

TABLE 4 | Parameters of the sigmoidal equation[a] used to estimate the effective mean dose of iodosulfuron-methyl-sodium (ALS inhibitor) required to reduce the fresh weight (GR_{50}) and plant survival (LD_{50}) by 50% in multiple-resistant (R) and -susceptible (S) populations of *Lolium* species in Chile.

Species	Population	d	b	R^2	Mean dose	RF	*P*-value
Growth reduction (GR_{50})							
L. multiflorum	R	99.8	1.4	0.99	50.2	12.9	<0.0001
	S	100.0	1.9	0.99	3.9		
L. perenne	R	102.7	1.0	0.99	27.0	9.0	<0.0001
	S	101.2	1.9	0.99	3.0		
L. rigidum	R	100.4	1.2	0.98	66.8	23.9	<0.0001
	S	101.3	1.6	0.99	2.8		
Plant survival (LD_{50})							
L. multiflorum	R	100.4	2.9	0.99	64.1	11.1	<0.0001
	S	101.5	1.7	0.99	5.8		
L. perenne	R	100.4	2.6	0.99	36.0	7.3	<0.0001
	S	101.6	1.7	0.99	4.9		
L. rigidum	R	99.5	2.3	0.98	82.8	13.4	<0.0001
	S	99.2	1.7	0.98	6.2		

[a] *Y = d/1+(x/g): where Y = percentage of fresh weight with respect to the untreated control, d = upper limit, b = slope of the curve, and g = herbicide concentration at the inflection point (i.e., GR_{50} or LD_{50}), and x = herbicide concentration. Resistance factors (RF = R/S) are the ratio of R to-S GR_{50} or LD_{50}. P is the level of significance of the non-linear model.*

Resistance to EPSPS Inhibitor

Among grasses worldwide, *Lolium* species have evolved the greatest number of cases of selection for glyphosate resistance (Heap, 2020). In Chile, the first case of glyphosate resistance was found in *L. multiflorum* in 2001 in fruit orchards in Region VIII (San Bernardo and Olivar) (Perez and Kogan, 2003), where two R populations presented RF 2- to 4-fold higher in relation to a S population. Subsequently, glyphosate resistant populations of the same species were also found in wheat fields of Region IX (Vilcún) with RF of 7.3 (Michitte et al., 2005). Glyphosate resistance levels of the R *L. multiflorum*, *L. perenne*, and *L. rigidum* populations included in this study, which varied between 6- and 11-fold based on growth reduction, and 4- to 9-fold according to plant mortality, were closer to those observed in the R *L. multiflorum* population of Vilcún, as well as to those reported for other glyphosate-resistant populations of *Lolium* spp. (Chandi et al., 2013; Fernández et al., 2017; Fernández-Moreno et al., 2017a,b; Yanniccari et al., 2017).

Herbicide resistance can be conferred by target-site resistance (TSR) and non-target site resistance (NTSR) mechanisms (Gaines et al., 2020). In this study we did not evaluate NTSR mechanisms; however, this does not mean that they cannot be involved in the herbicide resistance observed in the three R *Lolium* populations. Target site mutations at the Pro-106 position of the *EPSPS* gene in *Lolium* species, with Pro substituted by Ser, Thr, or Ala, impart low to intermediate glyphosate resistance levels, with RF ranging from 2 to 15 (Preston et al., 2009). Mutations in this position (Pro-106) narrow the cavity of the glyphosate binding site with EPSPS but maintain the affinity of this enzyme with its substrate (phosphoenolpyruvate), which allows plants exposed to glyphosate field doses to survive (Funke et al., 2006). The mutations found in the *EPSPS* gene of the R populations of *L. multiflorum* (Pro-106-Ser/Ala) and *L. rigidum* (Pro-106-Ser) explain their glyphosate resistance levels, as corroborated in the enzyme activity assays. The occurrence of multiple mutations among R individuals of *L. multiflorum* suggests a complex evolutionary history of the resistance traits, as observed in R *L. multiflorum* populations of northwest California

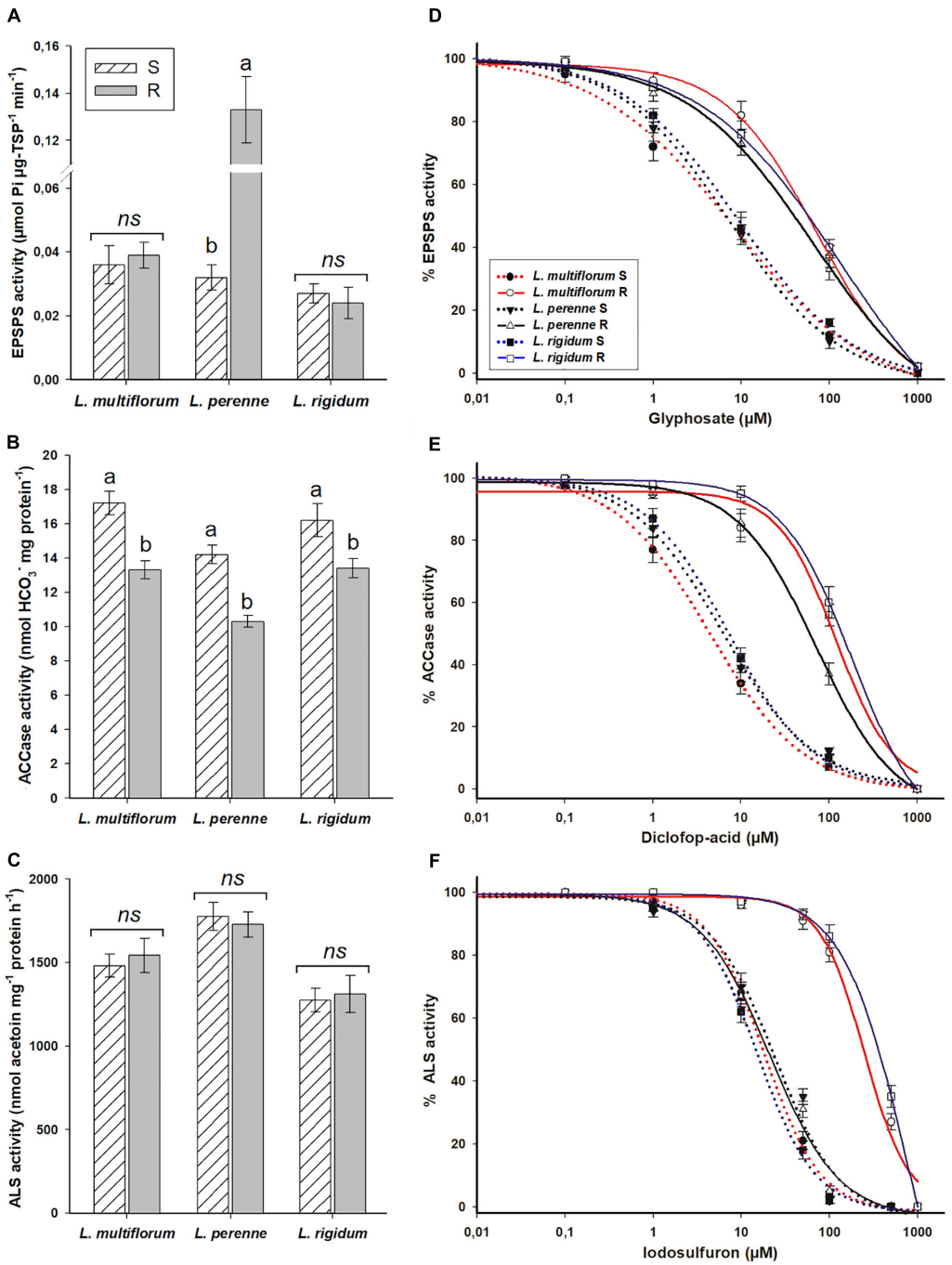

FIGURE 3 | Enzyme activity of resistant (R) and susceptible (S) populations of *Lolium* species in Chile, without and with presence of EPSPS **(A,D)**, ACCase **(B,E)** and ALS **(C,F)** inhibitors. Different letters above bars indicate differences between populations within species according to the Tukey test at 95%. NS, not significant. Vertical bars represent ± SE (n = 3).

that showed high nucleotide diversity at Pro-106 position between and within populations (Karn and Jasieniuk, 2017). Although *L. perenne* did not present any mutation, its glyphosate resistance could also be explained at target site level, since the R population presented an EPSPS specific activity four times higher than the S population. The high EPSPS specific activity in this R population suggests that there was an overproduction of the EPSPS enzyme, which could be due to increased *EPSPS* gene copy number, or alternatively EPSPS activity could be higher in R populations due to post-translational modifications of the enzyme (Gherekhloo et al., 2017; Gaines et al., 2019). Thus, although the EPSPS of R plants is glyphosate sensitive, higher

TABLE 5 | Parameters of the sigmoidal equation[a] used to estimate the concentrations (μM) of glyphosate, diclofop-methyl and iodosulfuron-methyl-sodium to inhibit the enzymatic activity of the EPSPS, ACCase and ALS by 50% (I_{50}), respectively, in multiple-resistant (R) and -susceptible (S) populations of *Lolium* species in Chile.

Species	Population	D	b	R^2	I_{50}	RF	*P*-value
EPSPS (5-enolpyruvylshikimate-3-phosphate synthase)							
L. multiflorum	R	99.9	14.3	0.98	102.3	14.2	<0.0001
	S	102.2	4.0	0.99	7.2		
L. perenne	R	101.6	2.9	0.99	65.3	8.4	<0.0001
	S	102.0	3.1	0.99	7.8		
L. rigidum	R	100.3	16.3	0.98	120.7	12.3	<0.0001
	S	100.6	5.2	0.99	9.8		
ACCase (acetyl-coenzyme A carboxylase)							
L. multiflorum	R	99.4	9.6	0.99	127.6	27.7	<0.0001
	S	101.6	2.0	0.99	4.6		
L. perenne	R	99.5	8.9	0.99	68.6	11.1	<0.0001
	S	100.6	2.7	0.98	6.2		
L. rigidum	R	99.4	11.5	0.99	186.2	29.6	<0.0001
	S	100.3	2.2	0.99	6.3		
ALS (acetolactate synthase)							
L. multiflorum	R	100.6	10.5	0.99	245.0	13.4	<0.0001
	S	99.5	6.9	0.99	18.3		
L. perenne	R	99.5	10.8	0.99	22.3	0.9	<0.0001
	S	98.3	8.8	0.98	24.0		
L. rigidum	R	100.4	8.6	0.99	392.5	23.2	<0.0001
	S	98.7	7.1	0.99	16.9		

[a] *$Y = d/1+(x/g)$: where Y = percentage of enzyme inhibition with respect to the specific activity, d = upper limit, b = slope of the curve, and g = herbicide concentration at the inflection point (i.e., I_{50}). Resistance factors (RF = R/S) are the ratio of R-to-S I_{50}.*

TABLE 6 | Frequency of amino acid substitutions (% and number of plants in parenthesis) in the genes of the target enzymes 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), acetyl-coenzyme A carboxylase (ACCase) and acetolactate synthase (ALS) of multiple-resistant (R) *Lolium* spp. populations from Chile.

Gene	Position	Amino acid change	*L. multiflorum*	*L. perenne*	*L. rigidum*
EPSPS	Pro-106	Ser	40 (8)	N.D.	60 (12)
		Ala	25 (5)	N.D.	N.D.
ACCase	Ile-1781	Leu	N.D.	N.D.	25 (5)
	Ile-2041	Asn	20 (4)	N.D.	N.D.
	Asp-2078	Gly	25 (5)	35 (7)	40 (8)
ALS	Pro-197	Ser	N.D.	N.D.	20 (4)
		Gln	N.D.	N.D.	15 (3)
	Trp-574	Leu	55 (11)	N.D.	30 (6)

Twenty plants were analyzed per population. No mutations were detected in the S populations and therefore, information is not shown. N.D., not detected.

concentrations of herbicide are required to completely inhibit it (Gaines et al., 2019). *EPSPS* gene amplification was reported as the main TSR mechanism in glyphosate-resistant populations of *L. multiflorum* in the United States (Salas et al., 2012), and *L. perenne* in Argentina (Yanniccari et al., 2017), conferring varying levels of resistance depending on the numbers of copies of the *EPSPS* gene of each population.

Resistance to ACCase Inhibitors

Resistance to ACCase inhibitors based on *ACCase* gene overexpression is unusual, and it has only been found to confer resistance in *Digitaria sanguinalis* in Canada (Laforest et al., 2017). In our case we can rule out this mechanism, since the ACCase specific activity of the three R *Lolium* spp. populations was slightly lower than in the S populations. This lower specific activity could be due to the Asp-2078-Gly mutation that was found in some individuals of the R populations of *L. multiflorum*, *L. perenne*, and *L. rigidum*. Unlike other mutations, which also confer resistance to ACCase inhibitors such as those found in another R *Lolium* individual (Ile-1781-Leu and Ile-2041-Asn), the Asp-2078-Gly mutation has a fitness penalty for the plants that carry it, which reduces the speed in catalyzing the formation of malonyl-CoA at the expense of natural substrates (acetyl-CoA, ATP, and HCO_3) (Vila-Aiub et al., 2015). This fitness penalty was observed in *Alopecurus myosuroides* and *L. rigidum* resistant to ACCase-inhibitors, where the specific activity of the ACCase of homozygous R plants of these weeds was reduced by ∼30% in relation to S plants (Vila-Aiub et al., 2015). The mutation Asp-2078-Gly has been reported to confer cross and different levels of ACCase resistance in *L. multiflorum* and *L. rigidum* around the world (Yu et al., 2007). In *L. perenne*, this mutation was found in R populations in Argentina (Yanniccari and Gigón, 2020). The selection of the Asp-2078-Gly mutation in the *ACCase* gene may fully explain the resistance to diclofop in *L. perenne*, and partially those observed in *L. multiflorum* and *L. rigidum* in dose-response and ACCase activity assays, since some individuals presented other mutations that also confer resistance to ACCase inhibitors.

The Ile-1781-Leu and Ile-2041-Asn combinations found in some R individuals of *L. multiflorum* and *L. rigidum*, respectively, contributed to an increase in the resistance to diclofop, but also showed that the R populations of these three *Lolium* species were not homogeneous. As already noted, because *Lolium* species are weeds of obligated cross-pollination, they may carry different alleles for both cross and multiple target-site resistance within populations (Malone et al., 2013; Martins et al., 2014), and in some case, in the same individual (Yu et al., 2007), as observed in clethodim-resistant *L. rigidum* in Australia, where different individuals of nine populations presented multiple ACCase mutations (one population presented 5 mutations), and two ACCase resistant alleles were found in single *L. rigidum* plants of two populations (Saini et al., 2015). The amino acid substitution Ile-1781-Leu is the most common mutation in resistant grass weed species and confers resistance to all classes of ACCase inhibitors (Kaundun, 2014), and does not have a fitness cost (Vila-Aiub et al., 2015). The Ile-2041-Asn mutation may or not confer resistance to ACCase inhibitors, therefore its presence does not imply it. For example, this mutation was reported to confer resistance to clethodim in *L. rigidum* (Yu et al., 2007), and *Phalaris paradoxa* (Hochberg et al., 2009),

but not in *A. myosuroides* (Délye et al., 2008). When the Ile-2041-Asn contributes to ACCase resistance, it is responsible for moderate to high resistance levels to ariloxyphenoxypropionates (FOPs) and low to moderate to phenylpyrazolines (DENs) (Ghanizadeh et al., 2019).

Resistance to ALS Inhibitors

Like for the *ACCase* gene, overexpression of the *ALS* gene is also rare (Zhao et al., 2018). In addition, *ALS* gene overexpression may be insufficient to provide resistance to ALS inhibitors (Wang et al., 2019). Since the specific activity of the ALS was similar between S and R populations within each *Lolium* species, the possible involvement of *ALS* gene overexpression in the resistance to iodosulfuron was ruled out. *ALS* gene sequencing revealed target site mutations in *L. multiflorum* (Trp-574-Leu) and *L. rigidum* (Pro-197-Ser/Gln + Trp-574-Leu). Although multiple mutations that confer resistance to ALS inhibitors in the same individual can be found (Singh et al., 2019), it is important to note that the two mutations found in *L. rigidum* occurred in different individuals. Mutations at Pro-197 (resistance to sulfonylureas) and Trp-574 (cross resistance to imidazolines and sulfonylureas) positions have been found in more than 20 weed species. These are the most common mutations conferring resistance to ALS inhibitors (Moss, 2017), since mutations at these points do not represent a major fitness cost (Yu and Powles, 2014). In *Lolium* species, six different substitutions have been found at these amino acid positions (Pro-197-Ala/Arg/Gln/Leu/Ser/Thr and Trp-574-Leu), conferring moderate to high resistance levels to ALS inhibitors in *L. multiflorum* and *L. rigidum* (Yu et al., 2008; Liu et al., 2014), as well as in others weeds such as *Descurainia sophia* (Deng et al., 2017), and *Rapistrum rugosum* (Ntoanidou et al., 2019).

The Asp-376-Glu and Trp-574-Leu mutations conferred resistance to ALS inhibitors in *L. perenne* (Menegat et al., 2016); however, we found no evidence of their participation in the iodosulfuron resistance of the R *L. perenne* population, which was confirmed by means of the dose-response assays, i.e., TSR mechanisms were not involved in such resistance in this species. Therefore, *L. perenne* resistance to iodosulfuron could presumably be governed by NTSR mechanisms, mainly enhanced herbicide metabolism mediated by major enzyme families of plant-degrading routes such as cytochrome P450, glutathione-*S*-tranferase and/or glycosyltransferase (Jugulam and Shyam, 2019). Considering that the occurrence of both TSR and NTSR mechanisms (mainly herbicide metabolism) in ALS- and ACCase-resistant weeds is widespread (Han et al., 2016; Fang et al., 2019), we cannot rule out the participation of NTSR mechanisms in the resistance of *L. multiflorum* and *L. rigidum*. In addition, some of the mutations found in the ALS and ACCase genes of the R *Lolium* populations from Chile have been reported to confer cross resistance (Murphy and Tranel, 2019). Therefore, future research will be focused in characterizing the NTSR-based iodosulfuron resistance in *L. perenne*, the potential contribution of these mechanisms in the R populations of *L. rigidum* and *L. multiflorum*, as well as possible cross resistance within chemical families of ALS and ACCase inhibitors.

CONCLUSION

An accumulation of target site mutations that confer resistance to ALS, ACCase and EPSPS inhibiting herbicides was found in R populations of *L. rigidum* and *L. multiflorum* collected in barley and wheat fields in Chile. The resistance profile to glyphosate, diclofop and iodosulfuron of *L. perenne* differed in relation to the other two species of *Lolium* (annual weeds), possibly due to its perennial habit facilitating vegetative reproduction instead of sexual reproduction. Therefore, in addition to including non-chemical methods in the management of these resistant *Lolium* species, the characterisation of the possible participation of NTSR-based herbicide resistance is essential for the establishment of a properly integrated weed management program. The Chilean farmers should implement crop rotation, the use of mechanical control, sowing variation, and others.

This is the first study in which herbicide resistance mechanisms were characterized in *L. rigidum* from South America. In addition, it is also the first time that multiple resistance to ACCase, ALS and EPSPS inhibiting herbicides have been reported in *L. multiflorum* and *L. perenne* worldwide.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JV-G, CP-B, AR-D, HC-H, and RD performed the dose-response and enzyme activity assays. RA, JT, and FB carried out the molecular analysis. RD performed funding project administration and supervision. All authors wrote, corrected and approved the submitted version of this manuscript.

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SUPPLEMENTARY MATERIAL

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Cytochrome P450 Herbicide Metabolism as the Main Mechanism of Cross-Resistance to ACCase- and ALS-Inhibitors in *Lolium* spp. Populations From Argentina: A Molecular Approach in Characterization and Detection

Marcos Yanniccari[1], Ramón Gigón[2] and Adelina Larsen[3]*

[1] Consejo Nacional de Investigaciones Científicas y Técnicas, Laboratory of Biotechnology and Plant Genetics, Chacra Experimental Integrada Barrow (MDA-INTA), Tres Arroyos, Argentina, [2] Private Consultant in Weed Control, Tres Arroyos, Argentina, [3] Instituto Nacional de Tecnología Agropecuaria, Laboratory of Biotechnology and Plant Genetics, Chacra Experimental Integrada Barrow (MDA-INTA), Tres Arroyos, Argentina

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Joel Torra,
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University of Córdoba, Spain
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***Correspondence:**
Marcos Yanniccari
marcosyanniccari@conicet.gov.ar;
marcosyanniccari@gmail.com

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Knowledge about the mechanisms of herbicide resistance provide valuable insights into evolving weed populations in response to selection pressure and should be used as a basis for designing management strategies for herbicide-resistant weeds. The selection pressure associated with reactive management against glyphosate-resistant *Lolium* spp. populations would have favored the herbicide resistance to ACCase- and ALS-inhibitors. This work was aimed to determine the sensitivity of 80 Argentinean *Lolium* spp. populations to ALS- and ACCase-inhibitor herbicides for use in wheat or barley and to study the mechanisms of resistance involved. Sensitivity to pinoxaden and iodosulfuron-mesosulfuron were positively correlated (r = 0.84), even though both affect different target sites. Inhibitors of cytochrome P450 monooxygenases (P450s) increased the sensitivity to pinoxaden and iodosulfuron-mesosulfuron in 94% of herbicide-resistant populations and target-site ACCase resistance mutations were detected only in two cases. Polymorphic variants were obtained with a pair primer designed on P450 sequences, cluster analysis discriminated around 80% of susceptible and P450-metabolic resistant plants sampled from a single population or different populations. Five markers corresponding to herbicide sensitivity were identified to be significantly associated with phenotypic variance in plants. Resistance to ALS- and ACCase-inhibitor herbicides were closely related, challenging the rotation of herbicides of both sites of action as a practice against resistance. In that sense, the use of pinoxaden and iodosulfuron-mesosulfuron would have provoked a selection on P450 genes that conduced a convergence of P450-metabolism based resistant *Lolium* spp. populations, which was detected by markers in a contribution to elucidate the molecular basis of this type of resistance.

Keywords: ryegrass, marker-trait associations, multiple-resistance, non-target site resistance (NTSR), target-site resistance (TSR)

INTRODUCTION

The weeds of farm land are a response to the management applied in the last years (Neve et al., 2009) because farming practices impose selective process on the weed community, conducing shifts in species composition or populations (Darmency, 2019). Thus, herbicide resistance is considered an evolutionary process, where the least herbicide-sensitive plants show an advantage in an environment with herbicide use (Délye et al., 2013). These select plants can involve generalist and/or specialist herbicide adaptations associated to the mechanisms of resistance. Non-target site mechanisms include metabolism or exclusion of the herbicide from the target and they have often been linked to generalist resistance, instead target-site mechanisms have been associated to specialist resistance, including amino acid substitutions, that affect the binding of the herbicide at the target enzyme or overexpression of the target site (Gaines et al., 2020).

Detoxification mechanisms could implicate the most problematic issue for weed management because it often involves unexpected resistance to alternative herbicides or yet undiscovered active principles (Preston, 2004; Yu and Powles, 2014). Herbicide metabolism seems to be controlled by multiple genes encoding enzyme systems, such as cytochrome P450 monooxygenases (P450s) and glutathione S-transferases, responsible for detoxifying non-chemically similar herbicides (Yuan et al., 2007; Yu and Powles, 2014). Specifically, P450s catalyze hydroxylation or dealkylation reactions related to the metabolism of non-related herbicides, such as ACCase- and ALS-inhibitors and glutathione S-transferases, that have been involved in reactions of conjugation to glutathione, directly processing the active herbicide or after the activity of other enzymes, such as P450s (Gaines et al., 2020). P450s conform the largest family of enzymes in plant metabolism, where represent around 1% of the protein-coding genes (Nelson and Werck-Reichhart, 2011). Although the role of P450s in herbicide resistant weeds has been well documented, the genes involved in P450-mediated resistance remained unknown for more than two decades until the detection of two P450 genes (CYP81A12 and CYP81A21) associated with resistance to ALS-inhibitors in *Echinochloa phyllopogon* (Iwakami et al., 2014). Recently, CYP81A genes involved in the detoxification of ACCase-inhibitors have been detected in multiple herbicide resistant *E. phyllopogon* (Iwakami et al., 2019). Moreover, P450 genes and others linked to the metabolism of herbicides have been associated with the increased activity of herbicide detoxification in *Lolium* spp. (Gaines et al., 2014; Duhoux et al., 2017).

Among the most troublesome herbicide-resistant weeds of the world, *Lolium* spp. populations have evolved resistance to at least seven sites of action (Heap, 2020) and new cases of herbicide-resistance are continually detected (Brunton et al., 2019). In Argentina, glyphosate-resistant *Lolium* spp. populations were recorded a decade ago in response to ACCase- and ALS-inhibiting herbicides that have been used to manage these cases at preplant or post-emergence in wheat and barley crops (Yanniccari et al., 2012). However, the selection pressure associated with reactive management against glyphosate-resistant populations would have favored resistance to other principle actives (Yanniccari and Gigón, 2020). In this sense, control failures of *Lolium* spp. with ACCase- and ALS-inhibiting herbicides have been observed frequently in the last few years (Vigna et al., 2017; Gigón and Yanniccari, 2018). This reduces the number of herbicides available for the control of the weed.

Lolium perenne ssp. *perenne* and *Lolium perenne* ssp. *multiflorum* or hybrids between them occur in 40% of wheat and barley crops from the Argentinean Pampas (Istilart and Yanniccari, 2012; Scursoni et al., 2014; Yanniccari et al., 2015) and lead to crop yield losses of up to 55% (Scursoni et al., 2012; Gigón et al., 2017). Knowing the status of resistance in *Lolium* spp. populations to ALS and ACCase-inhibiting herbicides used in wheat and barley and the mechanisms of resistance involved could contribute to understanding the evolution of the weed as an input for design strategies of management. The aim of this work was to determine the sensitivity of *Lolium* spp. populations to post-emergence herbicides used in wheat, study the mechanisms of resistance involved and develop candidate P450s based markers associated to herbicide resistance.

MATERIALS AND METHODS

Plant Collection

In December 2015 and 2016, seeds were sampled from 80 *Lolium* spp. populations that had arisen in fallows, wheat, or barley fields in the south of Buenos Aires Province (**Supplementary Material**). A combination of non-random and random procedures were applied during the selection of sampling points (Beckie et al., 2000; Burgos et al., 2013). The criterion for selecting 17 fields was based on suspicions of herbicide-resistant *Lolium* spp. due to failures of weed control with glyphosate, ACCase-, or ALS-inhibiting herbicides, communicated by technical advisors. Around those sites, four to five *Lolium* spp. populations distanced at least 5 km from each suspicious population were selected at random. One hundred heads from 20 plants were collected at random in each field. Samples were stored under room conditions at 20–25°C for 30 days and later threshed to remove the seeds from the heads, obtaining a bulk sample per population.

Herbicide-Sensitivity Analysis

In August 2016, 2017, and 2018, seeds of each population were sown in 2-L pots filled with soil to obtain 30 plants per pot. The plants were grown outdoors in a completely randomized design and irrigated at least every 3 days. Iodosulfuron-mesosulfuron (50 and 7.8 g a i L^{-1}, respectively, Hussar®, Bayer S.A.) and pinoxaden (50 g a i L^{-1} plus cloquintocet-mexyl 12.5 g L^{-1}, Axial®, Syngenta Agro S.A.) were applied to plants with 2 to 4 expanded leaves at recommended doses (12–1.87 and 40 g a i ha^{-1}, respectively) using a precision sprayer calibrated to deliver 200 L ha^{-1}. According to manufacturers' recommendations, 0.2% v/v ethoxylated alcohol was used as a surfactant for iodosulfuron-mesosulfuron spraying. Four untreated pots per population were maintained as controls and

four replicates of each herbicide treatment were performed, wherein each pot was a sampling unit.

Plant survival was evaluated after 45 days of herbicide applications, recording the percentage of plants whose growth or color of their leaves was similar to control plants (the number of surviving plants was divided by the total number of plants per pot and multiplied by 100). At the end of the plant life cycle, the production of seeds was checked in pots where surviving plants had previously been registered in order to verify compliance with the definition of herbicide resistance.

A correlation analysis was carried out processing data of plant survival to iodosulfuron-mesosulfuron and pinoxaden with GraphPad Prism® v. 6.01 (GraphPad Software, San Diego, CA, United States). The experiment was replicated twice.

Analysis of Mechanisms of Resistance

Based on plant survival to iodosulfuron-mesosulfuron or pinoxaden, possible mechanisms of herbicide-resistance were analyzed on populations when ≥40% of plants survived to one or both herbicides:

Partial Sequencing of *ACCase* and *ALS* Genes

Total DNA was extracted from five survival plants of each herbicide-resistant population (≥40% of plants survived to one or both herbicides). For that, the CTAB-DTT protocol was carried out following Doyle and Doyle (1990). DNA yield and quality were evaluated spectrophotometrically. The DNA was used as a template to amplify regions of the *ACCase* and *ALS* gene sequences. Two primer pairs (ACCase A (371bp): F 5′-TATGGCTGCAAACTCTGGTG-3′ and R 5′-GTATGCACCGTATGCCAAGT-3′; ACCase B (720bp): F 5′-GGCTCAGCTATGTTCCTGCT-3′ and R 5′-CAAGCCTACCCATGCATTCT-3′) were used according to Matzrafi et al. (2014) and two primer pairs (ALS122-205 (491bp): F 5′-GGGCGCCGACATCCTCGTCG-3′ and R 5′-ATCTGCTG CTGGATGTCCTT-3′; ALS197-574 (1396bp): F 5′-ACTCCAT CCCCATGGTGGC-3′ and R 5′-ATAGGCAGCACATGCTCC TG-3′; ALS574-653 (532bp): F 5′-TGGGCGGCTCAGTA TTACAC-3′ and R 5′-TCCTGCCATCACCTTCCATG-3′) were used following Yu et al. (2008). The PCR amplified fragments were sequenced by Macrogen service (Macrogen Inc., Seoul, South Korea) and the sequence data were cleaned, aligned, translated, and compared at the positions of all known resistance-conferring ACCase and ALS mutations (*ACCase*: Ile-1781, Trp-1999, Trp-2027, Ile-2041, Asp-2078, Cys-2088, and Gly-2096 codons and *ALS*: Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653 and Gly-654) using Chromas v.2.6.4 and Bioedit v.7.2. The nucleotide sequences obtained from D-P29 were queried using BLAST of the National Center for Biotechnology Information (NCBI).

Detoxification Through Cytochrome P450 Monooxygenases

Herbicide detoxification was evidenced using P450s inhibitors, such as malathion, 1-aminobenzo-triazole (ABT), and piperonyl butoxide (PBO; Matzrafi et al., 2014; Keith et al., 2015). Based on this, the interaction between iodosulfuron-mesosulfuron or pinoxaden and P450s' inhibitors was evaluated on plumule growth according to Yanniccari and Gigón (2020). A susceptible population (CP-P16) was used as a negative control (Yanniccari et al., 2018). Three grams of seeds of each population were incubated in petri dishes containing a wet filter paper in a growth chamber (75 mmol m^{-2} s^{-1} of photosynthetically active radiation, photoperiod of 12 h, and temperatures of 25 and 20°C for day and night, respectively). After 48 h, germinated seeds with a radicle length of ≥0.2 mm were transferred to 10 mL glass test tubes (four seeds per tube) containing cotton and 1 mL of one of the following treatments: (1.1) deionized water without herbicides or P450 inhibitors (control), (1.2) 10 ppm malathion, (1.3) 10 ppm ABT, (1.4) 20 ppm PBO, (2.1) 1 μM pinoxaden, (2.2) 1 μM pinoxaden + 10 ppm malathion, (2.3) 1 μM pinoxaden + 10 ppm ABT, (2.4) 1 μM pinoxaden + 20 ppm PBO, (3.1) 1 ppm iodosulfuron-mesosulfuron, (3.2) 1 ppm iodosulfuron-mesosulfuron + 10 ppm malathion, (3.3) 1 ppm iodosulfuron-mesosulfuron + 10 ppm ABT, and (3.4) 1 ppm iodosulfuron-mesosulfuron + 20 ppm PBO. Ten tubes were used as replicates for each population and treatment. After incubation for 5 days in a growth chamber under the conditions described above, the plumule length was measured from the point of attachment to the seed to the tip of the coleoptile.

A similar experiment was carried out to determine the response to P450 inhibitors in interaction with different herbicide doses (2, 5, 50, and 100 ppm of iodosulfuron-mesosulfuron or 2, 5, 50, and 100 μM of pinoxaden). This experiment was performed on those resistant populations that did not show response to the inhibitors at 1 ppm of iodosulfuron-mesosulfuron (2.2, 2.3, and 2.4) and 1 μM of pinoxaden (3.2, 3.3, and 3.4).

An ANOVA was performed to evaluate the differences among treatments. When ANOVA indicated significant effects, herbicide treatments with P450 inhibitors were compared to the respective herbicide treatment using Fisher's protected least significant difference test ($P < 0.05$). The experiment was replicated twice and data from both experiments were pooled when no significant differences between data sets were detected ($P > 0.05$). In all cases, residual plots indicated that variances were normally distributed and homogeneous.

Molecular Characterization of P450s in Herbicide-Resistant and Susceptible Plants

Susceptible and resistant plants were selected from a unique population (A-P13 with 71 and 63% of plant survival to iodosulfuron-mesosulfuron and pinoxaden respectively, **Supplementary Material**) in order to have a similar genetic background. Plants were grown for 8 weeks and vegetative clones of individual plants were propagated by tiller partition and repotted to obtain three ramets per plant. When individuals took root, each one was treated with recommended doses of pinoxaden (40 g a i ha^{-1}), iodosulfuron-mesosulfuron (12–1.87 g a i ha^{-1}), and deionized water as described above. After 45 days, plants were characterized as susceptible or herbicide-resistant (surviving plants to both herbicides). Following the

protocol described above, total DNA was isolated from untreated clones of 15 herbicide-resistant plants and 15 susceptible ones. Five primers were designed from consensus regions of 16 P450 sequences obtained by Fischer et al. (2001) and belonging to different cytochrome P450-families. Polymorphic variants on amplified band patterns were obtained with a primer pair (cyp450-F3 5′-TGGGCGATGTCGGTGCTG-3′ and cyp450-hemoR 5′-ACATATTCTAGGTCCCCATCCAAA-3′). The PCR conditions were initial denaturation at 94°C for 2 min and 30 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min, containing: 300 ηg DNA template, 0.4 μM of each primer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$, 1X reaction buffer (Inbio Highway), and 1 U Taq polymerase (Inbio Highway) in a 25 μL reaction mix. PCR products were separated by electrophoresis in 1.2% agarose gel stained with ethidium bromide in 0.5× tris-borate-ethylenediaminetetra acetic acid buffer. The results were interpreted using GelAnalyzer® 19.1 and amplified bands were scored as present (1) or absent (0), where bands of an identical molecular weight were considered a similar marker. Genetic similarities were calculated using the Jaccard coefficient and a dendrogram was built from similarities data by unweighted pair group method with arithmetic mean (UPGMA) using the software Infostat® 2017p. GLM association analyses incorporating pairwise kinship information as a covariate were performed employing the R® Core Team 3.6.2 package Genome Association and Prediction Integrated Tool (GAPIT; Lipka et al., 2012). The *P*-values were separately tested using the false positive discovery rate (FDR) test with R® Core Team 3.6.2.

Randomizing the genetic background, a similar experiment was carried out from genomic DNA extracted from 15 herbicide-resistant plants (surviving to pinoxaden or iodosulfuron-mesosulfuron treatments) and 15 susceptible individuals (untreated plants from susceptible accessions) of different populations taken at random within both groups. Data analysis was performed following the methodology detailed above.

RESULTS

Herbicide-Sensitivity Analysis

Thirty-five *Lolium* spp. populations showed a percentage of pinoxaden- or iodosulfuron-mesosulfuron-resistant plants higher than 40%. An association between sensitivity to both herbicides was detected with a correlation coefficient of 0.84 (**Figure 1**). Three populations (MH-P33, D-P35, and TA-P41) showed a high proportion (≥75%) of herbicide-resistant plants to one herbicide and a lower percentage of resistance (≤50%) to another principle active (**Supplementary Material**). In most cases, the differences between plant survival percentages were lower than 30 points (**Figure 1**).

Mechanisms of Herbicide-Resistance

No evidence of target-site mechanisms of ALS-inhibitor-resistance were found analyzing the codons Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654. However, plants of two populations showed target-site mutations associated with pinoxaden-resistance Asp-2078-Gly in the TA-P41 population (detailed in Yanniccari and Gigón, 2020) and Cys-2027-Trp in D-P29. In the last case, a transversion was detected on the third base of the codon and the mutation was found in a heterozygous state (**Figure 2**).

Firstly, the response to P450 inhibitors was detected in 28 populations and depending on the inhibitor used plant responses are rather different among populations (**Table 1**). Malathion, ABT, or PBO increased the sensitivity to pinoxaden and iodosulfuron-mesosulfuron in 28 cases but in two populations (VE-P0 and D-P29), inhibitors did not affect the response of plumule growth to pinoxaden although it increased the damage

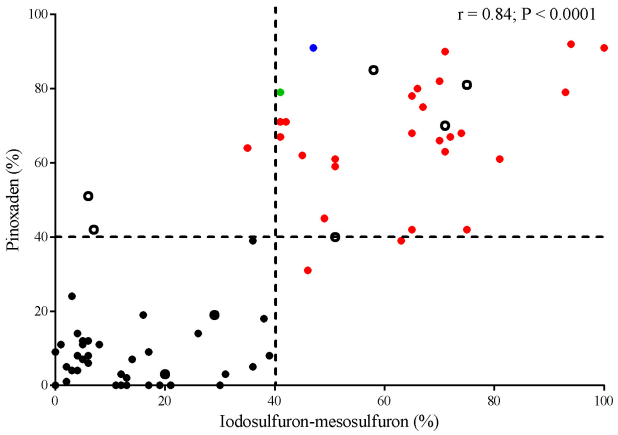

FIGURE 1 | Correlation between plant survival of *Lolium* spp. populations to iodosulfuron-mesosulfuron (ALS-inhibitor) and pinoxaden (ACCase-inhibitor). Discontinued lines indicate a threshold of 40% plant survival to each herbicide. P450-metabolism based resistant (red symbols), TA-P41 (blue symbol), and D-P29 (green symbol) populations. Non-evidence of target-site resistance or herbicide P450-metabolism (open symbols).

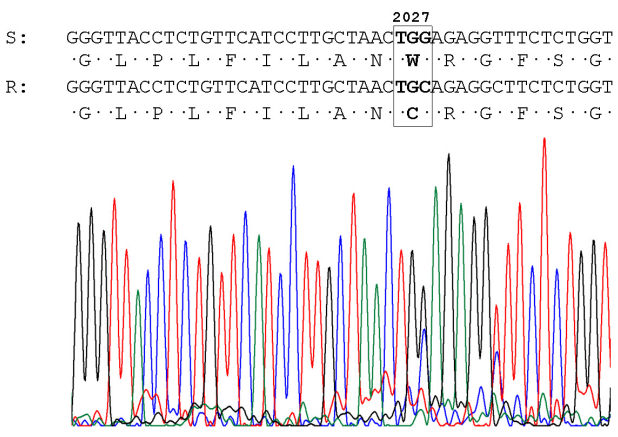

FIGURE 2 | Sequence and chromatogram of the *ACCase* gene obtained from the D-P29 (R) population and the conceptual translation of the amino acid sequence. The resistance-conferring codon is shown in the box. Numbers refer to amino acid positions of full-length ACCase in *Alopecurus myosuroides*.

TABLE 1 | Effects of iodosulfuron-mesosulfuron (1 ppm) and pinoxaden (1 μM) plus malathion (M), 1-aminobenzo-triazole (ABT), piperonyl butoxide (PBO), or without P450-inhibitors (WI) on plumule length of seedlings.

Population	Iodosulfuron-mesosulfuron				Pinoxaden			
	WI	M	ABT	PBO	WI	M	ABT	PBO
A-P13	95	89	52*	55*	44	25*	32	25*
F-P18	89	55*	86	80	35	30	12*	27
DR3	117	70*	65*	58*	70	65	46*	58
LC-P13	97	103	60*	56*	85	58*	81	48*
D-P19	92	88	66*	79	91	97	17*	23*
TA-P24	101	62*	60*	55*	93	59*	66*	57*
E-P32	86	58*	60*	50*	93	53*	47*	41*
MH-P33	90	93	62*	86	72	61	38*	66
D-P35	95	53*	55*	56*	82	42*	48*	47*
V-P39	91	60*	71*	65*	84	40*	49*	43*
P-46	85	83	80	90	70	77	75	67
VE-P0	75	49*	52*	46*	44	37	35	28
SC-P14	86	94	96	92	73	83	78	92
ST-P34	87	77	52*	75	79	73	42*	52*
BW-P12	89	87	51*	65*	48	45	24*	22*
EP-P26	88	80	58*	75	80	53*	42*	38*
CO-P15	103	60	64*	86	85	30*	27*	83
D-C19	88	75	50*	48*	86	96	28*	33*
GTA-P22	31	29	21	20	103	94	90	95
D-P275	93	79	81	44*	47	24*	32	24*
EP-P2	93	63*	61*	52*	87	52*	42*	38*
CAP-P11	36	31	27	38	94	105	109	93
BH-P11	94	105	34*	41*	83	36*	69	45*
TA-P41	49	34	43	37	99	100	99	90
D-P29	64	55	31*	34*	104	95	98	102
L-P4	96	52*	44*	42*	85	49*	50*	41*
LO-P6	92	51*	54*	78	107	36*	51*	82
TP-P14	64	72	58	35*	105	87	52*	59*
LO-P17	70	62	45*	43*	90	54*	45*	41*
LM-P23	98	114	107	111	93	97	103	107
DO-P25	79	57*	72	51*	92	53*	80	48*
LD-P27	86	94	57*	96	96	84	68*	100
DSM-P13	87	97	58*	50*	105	94	61*	55*
D-P15	76	83	53*	51*	90	95	63*	61*
LL-P25	83	79	91	86	88	88	95	85
CP-P16	28	30	26	19	24	19	22	17

*Mean values relatives to the treatment without herbicides or P450-inhibitors (%). *Indicate significant effects ($P \leq 0.05$) of P450-inhibitors compared to the respective control.*

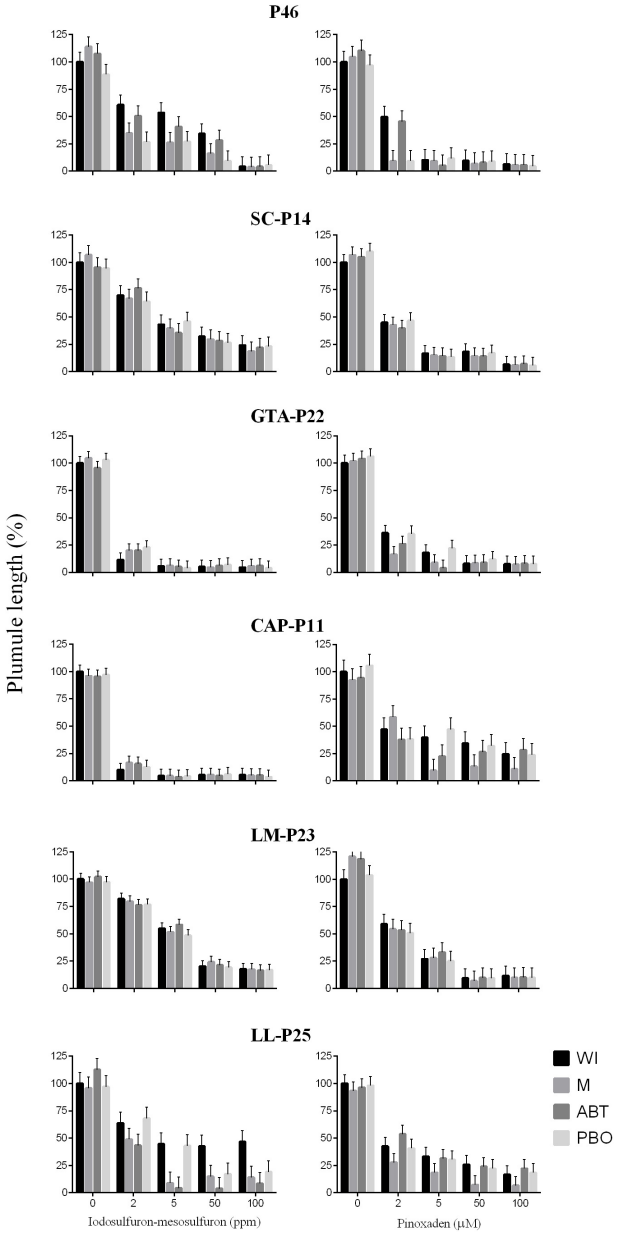

FIGURE 3 | Effects of different doses of iodosulfuron-mesosulfuron and pinoxaden plus malathion (M), 1-aminobenzo-triazole (ABT), piperonyl butoxide (PBO), or without P450-inhibitors (WI) on plumule length of seedlings. Bars represent mean values relatives to the treatment without herbicides or P450-inhibitors (%) and error bars indicate one standard error.

provoked by iodosulfuron-mesosulfuron. P450 inhibitors did not condition the response of seven herbicide-resistant populations (P-46, SC-P14, GTA-P22, CAP-P11, TA-P41, LM-P23, and LL-P15) to iodosufuron-mesosulfuron and pinoxaden at a dose of 1 ppm and 1 μM, respectively. 1-Aminobenzo-triazole increased the iodosulfuron-mesosulfuron-sensitivity in 24 cases and only two populations treated with this herbicide showed responses to malathion or PBO without recording a significant effect of ABT. In 26 cases, the action of pinoxaden was exacerbated when it was combined with PBO or ABT (**Table 1**). Neither P450 inhibitor significantly affected the plumule growth in absence of the herbicide (**Supplementary Material**). The low pinoxaden-sensitivity of P-46, GTA-P22, and CAP-P11 was reverted with P450 inhibitors at a dose of pinoxaden of >1 μM. In the same way, the response to P450 inhibitors was detected in P-46 and LL-P25 treated with 2–50 ppm and ≥5 ppm of iodosulfuron-mesosulfuron (**Figure 3**). For two resistant populations (SC-P14 and LM-P23), no evidence

of target-site resistance or herbicide P450-metabolism was detected (**Figure 3**).

Molecular Characterization of P450s in Herbicide-Resistant and Susceptible Plants

Polymorphic variants were obtained with a pair primer (cyp450-F3 and cyp450-hemoR), where 10 different bands (between 200 and 1950 bp) were detected at least in a frequency of 26% analyzing individuals from a single population (A-P13) or in 13% of plants from different populations.

Initially, a dendrogram was constructed on the basis of the similarity index among plants obtained from A-P13. A 50% cutoff value gave 11 distinct clusters where four groups (V, VI, VII, and VIII) were associated with 100% of herbicide-susceptible individuals. In contrast, 7 clusters (I, II, III, IV, IX, X, and XI) contained 86% of pinoxaden and iodosulfuron-mesulfuron-resistant plants (**Figure 4**). Then, when band patterns were analyzed, considering individuals from thirty populations, 9

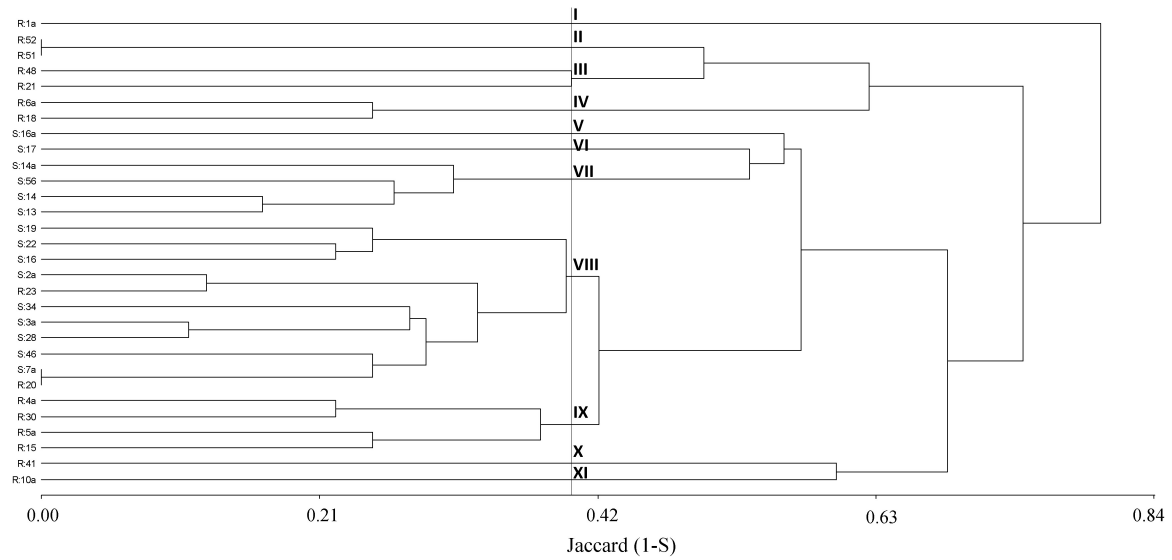

FIGURE 4 | Cluster analysis dendrogram based on ten markers (m1 to m10) detected on 15 herbicide-resistant (R) and 15 susceptible (S) plants from the A-P13 population. Description of clusters I to XII at a cutoff of 50%.

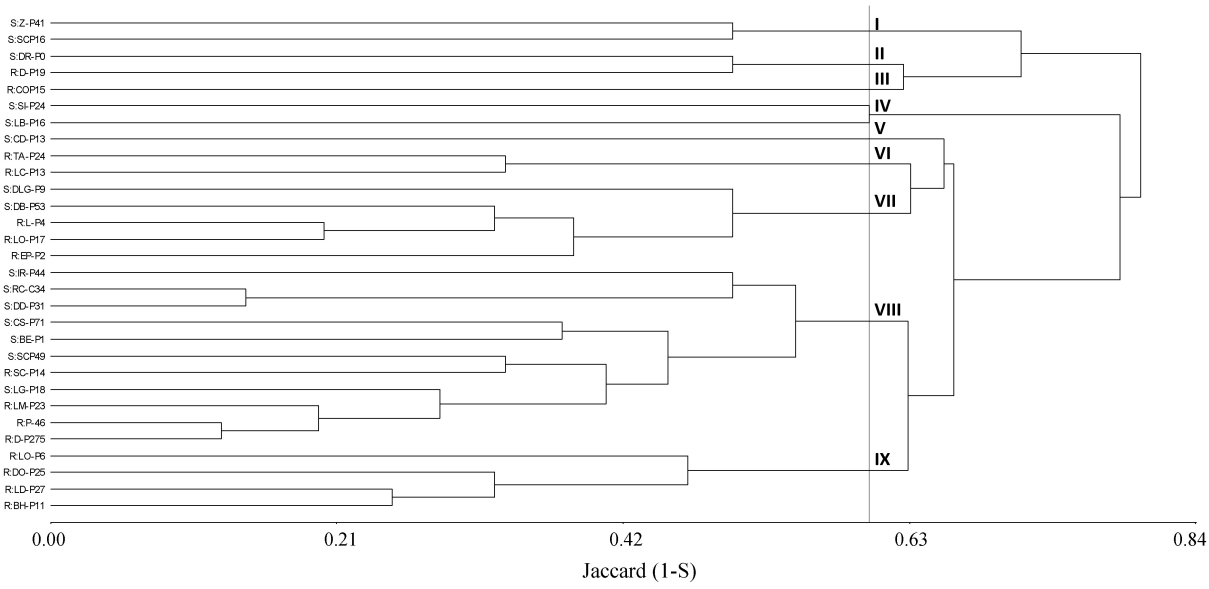

FIGURE 5 | Cluster analysis dendrogram based on ten markers (m1 to m10) detected on 15 herbicide-resistant (R) and 15 susceptible (S) plants from different populations. Description of clusters I to VIII at a cutoff of 60%.

clusters were obtained at a cutoff of 60%. Four clusters (I, V, VII, and VIII) grouped 80% of susceptible-individuals, where the biggest group (cluster VIII) included 7 susceptible plants and 4 herbicide-resistant individuals, of which, two were isolated from populations without evidence of P450 metabolism (SC-P14 and LM-P23, **Figures 3**, **4**). Herbicide-resistant individuals were mainly grouped into four clusters (III, VI, VII, and IX), containing 66% of the total resistant plants analyzed (**Figure 5**).

Both in the intra- and inter-population analysis, three markers were significantly associated with herbicide-sensitivity detected by GLM (**Table 2**). Of these markers, a band (m7) was linked to the susceptible response to pinoxaden and iodosulfuron-mesosulfuron in both types of analysis and two pair of markers (m2 and m5; m3, and m9) were significantly associated with susceptibility/resistance according to the experiment considered. In all cases, significant effects showed *P*-values $\leq$0.03 and FDR-adjusted *P*-values $\leq$0.10 (**Table 2**).

DISCUSSION

At a frequency of 40% of resistant plants, a farmer or a technical adviser can detect a failure in chemical control (Burgos et al., 2013) and this threshold was used to compare the herbicide-sensitivity of *Lolium* spp. populations. Notably, the responses of accessions to pinoxaden and iodosulfuron-mesosulfuron were positively correlated, even though both affect different target sites (**Figure 1**). Thus, populations with a high frequency of pinoxaden-resistant plants (>40%) were also linked to high iodosulfuron-mesosulfuron-resistance and vice-versa. P450-mediated herbicide metabolism seems to be the main mechanism of resistance detected among populations with a high percentage of resistant plants (**Figure 1**). In these cases, the metabolism of pinoxaden and iodosulfuron-mesosulfuron was reverted by one or more P450-inhibitor, however the three compounds (malathion, ABT, and PBO) were only effective inhibitors of both herbicides in six populations (TA-P24, E-P32, D-P35, V-P39, EP-P2, and L-P4; **Table 1**). Differential patterns of inhibition of herbicide metabolism by malathion, ABT and PBO could be associated to different metabolic systems, probably involving distinct isoenzymes of P450 (Preston et al., 1996). In four cases (P-46, GTA-P22, CAP-P11, and LL-P25) the P450 inhibitors action as synergists for iodosulfuron-mesosulfuron or pinoxaden was evidenced (**Figure 3**) at herbicide doses higher than those used to detect the P450-mediated metabolism in most herbicide-resistant populations (**Table 1**). It will be necessary to determine if an overexpression of P450 or particular isoenzymes are involved in these four populations as found by Iwakami et al. (2014). For SC-P14 and LM-P23 the resistance mechanism(s) are unknown.

P450-metabolism based resistant *Lolium spp.* populations would have evolved resistance under a selection process, tending to favor a generalist herbicide adaptation, despite guaranteeing an adequate rotation of ALS- and ACCase-inhibitor herbicides for use in wheat or barley. It supports the necessity of improving recommendations of herbicide rotations to avoid repeated selection with herbicides that are vulnerable to shared resistance mechanisms (Gaines et al., 2020).

P450 genes would have an important role in the evolution and diversification of organisms, providing adaptive advantages (Omura, 2013). In that sense, markers associated with P450 genes have been highlighted as an efficient tool to study genetic diversity in plants (Ravi et al., 2020). In the current results, cluster analysis discriminated around 80% of susceptible and P450-metabolic resistant plants sampled from a single population or different populations. In the latter case, ten herbicide-resistant populations were mainly grouped into VI, VII, and IX clusters (**Figure 5**). Interestingly, the three P450 inhibitors increased the iodosulfuron-mesosulfuron or pinoxaden sensitivity in herbicide-resistant populations grouped into VI and VII clusters (TA-P24, LC-P13, L-P4, LO-P17, and EP-P2), however, only one (ABT) or two (malathion and ABT or malathion and PBO) P450 inhibitors affected the herbicide metabolism in D-P275, LO-P6, DO-P25, and LD-P27 grouped into IX cluster (**Figure 5**). This pattern could support the hypothesis that different P450 isoenzymes are involved in several herbicide-resistant populations. The analyzed markers could be linked to resistance/susceptibility alleles or evidence of the effects of selection pressure on patterns of P450s polymorphism. Nelson and Werck-Reichhart (2011) have pointed out that P450s are an excellent mirror of plant evolution and its role in adaptation.

Beyond the ten markers considered in cluster analysis, through GLM, five markers, corresponding to herbicide sensitivity, including m2, m5, and m7, were identified to be significantly associated with phenotypic variance in plants obtained from the A-P13 population and m3, m7, and m9 explained herbicide sensitivity in individuals from 30 different populations (**Table 2**). In both experiments, the presence of the m7 band was significantly associated with the herbicide-resistant phenotype. The amplified fragment could

TABLE 2 | Marker-trait associations for herbicide-resistance in plants from the A-P13 population and different populations.

Marker	A-P13		Populations	
	P-value	FDR	P-value	FDR
m1	1.00	1.00	0.29	0.59
m2	**0.02**	**0.10**	0.72	0.72
m3	0.27	0.39	**0.01**	**0.07**
m4	0.07	0.13	0.16	0.40
m5	**0.03**	**0.10**	0.71	0.72
m6	0.06	0.13	0.71	0.72
m7	**0.01**	**0.10**	**0.01**	**0.07**
m8	0.08	0.13	0.45	0.68
m9	0.46	0.52	**0.02**	**0.07**
m10	0.46	0.52	0.47	0.68

P-values obtained by GLM analysis and results of false positive discovery rate (FDR) test are shown. P-values ≤ 0.05 and FDR-adjusted P-values ≤ 0.10 are in bold.

be part of one or more resistance genes, or the marker could have no functional role and it could be inherited together with resistance genes. In any case, the results provide the possibility to perform diagnostic prediction of P450s-mediated pinoxaden- and iodosulfuron-mesosulfuron-resistance, the most common mechanism of resistance detected in *Lolium* spp. populations.

Resistance to multiple herbicides emerges as a challenge for current and future weed management. Chemical control practices that seek to reduce selection for specialist resistance traits may promote the evolution of generalist resistance (Comont et al., 2020). The study of metabolic resistance mechanisms and the elucidation of a molecular basis is a difficult and arduous process but a better understanding of these generalist mechanisms will reinforce comprehension of the evolution of weed populations in response to selection pressures and contribute to the development of weed management strategies to delay resistance (Nandula et al., 2019). The findings of the current study indicate that generalist herbicide resistance is due to P450-mediated detoxification, which was highly frequent in *Lolium* spp. populations from Argentinean Pampas. Resistance to ALS- and ACCase-inhibitor herbicides were closely related, challenging the rotation of herbicides of both sites of action as a practice against resistance. Herbicide rotations should be designed to consider the most common mechanisms of resistance associated with each principle active to alternate herbicides commonly conditioned by the same mechanism. In that sense, the use of pinoxaden and iodosulfuron-mesosulfuron would have provoked selection of P450 genes that conduced a convergence of P450-metabolism based resistant in *Lolium* spp. populations, which was detected by markers in a contribution to elucidate the molecular basis of this type of resistance.

DATA AVAILABILITY STATEMENT

The datasets GENERATED for this study can be found in NCBI GenBank accessions TA-P41: MW178199,D-P29: MW178200.

AUTHOR CONTRIBUTIONS

MY and RG conceived, designed, and conducted the experiments. MY wrote the manuscript. All authors analyzed the data, provided editorial advice, and revised manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.600301/full#supplementary-material

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Diversified Resistance Mechanisms in Multi-Resistant *Lolium* spp. in Three European Countries

Laura Scarabel[1], Silvia Panozzo[1], Donato Loddo[1], Solvejg K. Mathiassen[2], Michael Kristensen[2], Per Kudsk[2], Thomas Gitsopoulos[3], Ilias Travlos[4], Eleni Tani[4], Dimosthenis Chachalis[5] and Maurizio Sattin[1]*

[1] Institute for Sustainable Plant Protection (IPSP-CNR), National Research Council of Italy, Padua, Italy, [2] Department of Agroecology, Aarhus University, Flakkebjerg, Denmark, [3] Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization-Demeter, Thessaloniki, Greece, [4] Department of Crop Science, Agricultural University of Athens, Athens, Greece, [5] Benaki Phytopathological Institute, Weed Science Laboratory, Athens, Greece

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Joel Torra, Universitat de Lleida, Spain

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Nilda Roma Burgos, University of Arkansas, United States

***Correspondence:**
Laura Scarabel
laura.scarabel@cnr.it

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Annual ryegrass species (*Lolium* spp.) infest cereal crops worldwide. Ryegrass populations with multiple resistance to the acetyl coenzyme A carboxylase (ACCase) and acetolactate synthase (ALS) inhibitors are an increasing problem in several European countries. We investigated the resistance pattern and level of resistance in ryegrass populations collected in Denmark, Greece and Italy and studied the diversity of mechanisms endowing resistance, both target-site and metabolism based. All populations showed high resistance indexes (RI) to the ALS inhibitors, iodosufuron-methyl-sodium + mesosulfuron-methyl (RI from 8 to 70), whereas the responses to the two ACCase inhibitors, clodinafop-propargyl and pinoxaden, differed. The Greek and Italian populations were moderately to highly resistant to clodinafop (RI > 8) and showed low to moderate resistance to pinoxaden (RI ranged from 3 to 13) except for one Italian population. In contrast, the Danish *Lolium* populations showed low to moderate resistance to clodinafop (RI ranged from 2 to 7) and only one population was resistant to pinoxaden. Different mutant *ACCase* alleles (Leu_{1781}, Cys_{2027}, Asn_{2041}, Val_{2041}, Gly_{2078}, Arg_{2088}, Ala_{2096}) and *ALS* alleles (Gly_{122}, Ala_{197}, Gln_{197}, Leu_{197}, Ser_{197}, Thr_{197}, Val_{205}, Asn_{376}, Glu_{376}, Leu_{574}) endowing resistance were detected in the Greek and Italian populations. In several plants, no mutated *ALS* and *ACCase* alleles were found showing a great heterogeneity within and among the Greek and Italian populations. Conversely, no mutant *ACCase* alleles were identified in the four Danish populations and only one mutant *ALS* allele (Leu_{574}) was detected in two Danish populations. The expression level of nitronate monooxygenase (*NMO*), glutathione *S*-transferase (*GST*) and cytochrome P450s (*CYP72A1* and *CYP72A2*) varied broadly among populations and individual plants within the populations. Constitutive up-regulation of *GST*, *CYP72A1* and *CYP72A2* was detected in resistant plants respect to susceptible plants in one Danish and one Italian population. It appears that the

mechanisms underlying resistance are rather complex and diversified among *Lolium* spp. populations from the three countries, coevolution of both target-site resistance and metabolic based herbicide resistance appears to be a common feature in Denmark and Italy. This must be considered and carefully evaluated in adopting resistance management strategies to control *Lolium* spp. in cereal crops.

Keywords: ryegrass, target-site resistance, enhanced gene expression, metabolism, multiple herbicide resistance

INTRODUCTION

Ryegrass species (*Lolium* spp.) are obligate out-crossers with high genetic variability and fecundity (Pedersen et al., 2007; Holt et al., 2013). They are common weeds in many European countries and infest numerous cropping systems, including cereal crops, where they are considered a threat for the sustainability of cereal production. Historically, the control of *Lolium* spp. has been carried out with herbicides inhibiting acetyl coenzyme-A carboxylase (ACCase) but several populations have evolved resistance to this herbicide group. It is recognized that the diversification of the selection pressure by using herbicides with different sites of action is a key point for resistance management. Hence, the subsequent registration of acetolactate synthase (ALS) inhibitors introduced another herbicide site of action (SoA) to overcome this problem. These herbicides, able to control broad-leaved weeds as well as some grass species including *Lolium* spp., have been widely used by cereal growers. The recurrent treatments with herbicides having the same SoA have selected resistant *Lolium* spp. populations and this significantly reduces the number of herbicides available to control these weed species.

Several resistance mechanisms have been reported for *Lolium* spp. Among them, gene mutations reducing or blocking herbicide binding by conferring amino-acid changes in a target protein (Target Site Resistance, TSR) and enhanced metabolism causing accelerated herbicide degradation (one of the non-target-site resistance, NTSR mechanisms) are the main mechanisms in grass weeds such as *Lolium* spp. (Powles and Yu, 2010; Délye et al., 2013). TSR has been extensively studied in the last 20 years and many of the known mutations endowing herbicide resistance in the *ALS* (Yu and Powles, 2014) and *ACCase* (Kaundun, 2014) genes have been found in *Lolium* spp. populations (Tan et al., 2007; Scarabel et al., 2011; Han et al., 2016). Early works have established the presence of metabolic resistance to diverse herbicides such as chlorsulfuron, chlorotoluron in *Lolium rigidum* populations from Australia (Cotterman and Saari, 1992; Preston et al., 1996) and also the presence of both TSR and enhanced metabolism-based resistance (hereinafter referred as EMR) mechanisms in the same plant (Christopher et al., 1992; Tardif and Powles, 1994). From then on, EMR has been understudied and only recently, it was recognized as the predominant type of resistance to ACCase- and ALS inhibitors in grasses (Gaines et al., 2020). EMR, differently to TSR, can confer cross-resistance to herbicides with different SoA, including herbicides to which weeds have not been previously exposed (Petit et al., 2010). EMR is considered to be polygenically inherited, involving multiple genes encoding for metabolic enzymes such as cytochrome P450 monooxygenase (P450), glucosyl transferases (GT), glutathione *S*-transferases (GST), esterases and ABC transporters (Yuan et al., 2007; Duhoux and Délye, 2013; Duhoux et al., 2017). Four consistently over-expressed genes were identified in resistant individuals of *Lolium rigidum*, a close relative of *Lolium multiflorum*. These included two *P450s*, one nitronate monooxygenase (*NMO*) and one *GT* (Gaines et al., 2014, 2020). In *L. multiflorum* higher expressions of these four metabolism-related genes were reported in individuals of resistant populations from Denmark (Mahmood et al., 2016).

In Greece, the first case of *L. rigidum* resistant to both diclofop-methyl (ACCase inhibitor) and chlorsulfuron (ALS inhibitor) was reported in 2000 (Kotoula-Syka et al., 2000). Subsequently, other populations resistant only to chlorsulfuron were found and the resistance mechanism was attributed to enhanced activity of P450 in some populations and to TSR in others (Kaloumenos et al., 2012). In Italy, the first ACCase-resistant *Lolium* spp. population was recorded in the mid-1990s in central Italy (Bravin et al., 2001). Since then, ACCase-resistant cases have also spread to northern Italy and a few years ago *Lolium* spp. populations resistant to both ALS and ACCase inhibitors were recorded (Collavo et al., 2013) and they are now increasing. In Denmark, herbicide resistance in *Lolium* spp. appeared later than in Italy and Greece with the first case of *Lolium multiflorum* resistant to an ALS-inhibitor (iodosulfuron) registered in 2010 (Mathiassen, 2014). Since then, cases have been increasing and the first case of multiple-resistance to ACCase and ALS inhibitors was reported in 2010 (Heap, 2020).

Lolium spp. is very prone to evolve resistance and multi-resistant cases are increasing in the three countries. It was also shown that metabolic resistance evolves rapidly in *L. rigidum* when herbicides are used at low or suboptimal doses and this is an important point to consider for weed management (Neve and Powles, 2005; Manalil et al., 2011). Efforts should therefore be made to limit the evolution of resistance and to ensure the sustainability of cereal crops production. The aims of this work were (1) to determine the level of resistance to ALS and ACCase inhibitors through bioassays in twelve *Lolium* spp. populations collected in Denmark, Greece, and Italy; (2) to investigate the resistance mechanisms involved, both the detection of *ALS* and *ACCase* alleles endowing TSR and the presence of EMR mechanism. For the latter purpose, the gene expression of four

herbicide metabolism related genes (*NMO*, *GST*, P450s *CYP72A1* and *CYP72A2*) was investigated through qPCR.

MATERIALS AND METHODS

Origins of *Lolium* Populations

Seeds from *Lolium* spp. plants were collected in winter cereal fields from three European countries (Denmark, Greece, and Italy) where the control of these grass species by ALS and ACCase inhibitors was poor. After a preliminary screening of the populations, conducted in each country, 12 populations, four from each country, with a high frequency of plants resistant to both ALS and ACCase inhibitors were selected (**Table 1**). Additionally, susceptible reference populations from each of the three countries were included. All seed samples were cleaned and stored in paper bags at 4°C until use.

Outdoor Dose-Response Experiments

Two whole-plant dose-response experiments were carried out at Legnaro (PD), Italy (45° 21′ N, 11° 58′ E). The experiments were performed as outdoor pot experiments during spring 2018 and autumn 2018 using commercial formulations of ALS and ACCase inhibitor herbicides.

To break dormancy, seeds were placed in Petri dishes on wet filter paper and vernalized at 4°C under dark conditions for 3 days. Then, seeds were placed in a germination cabinet and kept for 5 days at 25/15°C (day/night) with a 12 h photoperiod. Germinated seedlings at similar growth stages were transplanted into 16 cm diameter pots filled with a standard potting mixture (60% silty loam soil, 15% sand, 15% perlite, and 10% peat). The pots were placed outside in a semi-controlled environment and watered regularly to maintain the substrate at field capacity.

The experimental layout was a completely randomized design with three biological replicates (pots), each one with 9 seedlings. Herbicide application was carried out at BBCH 21-22 (Hess et al., 1997). All populations were treated with two ACCase inhibitors (pinoxaden and clodinafop-propargyl) and one of ALS inhibitor (mesosulfuron-methyl + iodosulfuron methyl-sodium) (**Table 2**). Herbicides were applied using a precision bench sprayer delivering 300 L ha^{-1} at a pressure of 215 kPa and speed of about 0.75 ms^{-1} with a boom equipped with three flat-fan (extended range) hydraulic nozzles (Teejet®, 11002). The herbicide doses applied ranging from 1/16 N to 4 N for the susceptible populations and from 1/8 N to 8 N for the resistant populations with N being the recommended field dose in Italy.

Four weeks after treatment, plant survival and shoot fresh weight were recorded for each pot and expressed as percentage of the mean of the non-treated control.

The dose-response data were analyzed using a non-linear regression analysis based on the log-logistic equation: $Y = C + [(D - C)/[1 + (x/I_{50})^b]$ where Y is the fresh weight or survival, C and D are the lower and upper asymptotes at very high and infinitely low doses, respectively, b is the slope of the curve around its inflection point, I_{50} is the dose giving a response equivalent to midway between the D and C parameters and x is the herbicide dose (g a.i.ha^{-1}) (Seefeldt et al., 1995).

The ED_{50} (herbicide dose causing 50% plant mortality), GR_{50} (herbicide dose causing 50% reduction in fresh weight) and relative standard errors were estimated using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, United States). For biological reasons and to improve the best-fit values of the parameters, the lower and upper asymptotes of plant survival and fresh weight data were constraints to 0 and 100%, respectively (Onofri, 2005). Data of each population and for each herbicide were analyzed together and an

TABLE 1 | Some details of *Lolium* spp. populations from Denmark, Greece, and Italy used in the study.

Country	Location of collection	*Lolium* species	Population code	Year of sampling	Selecting agents(HRAC group)
Denmark	Randers	*L. perenne*	DK6	2017	ACCase + ALS
	Sønderborg	*L. multiflorum*	DK29	2017	ACCase + ALS
	Løgumkloster	*L. multiflorum*	DK47	2017	ACCase + ALS
	Slagelse	*L. multiflorum*	DK90	2017	ACCase + ALS
	Skive	*L. multiflorum*	DK100[a]	2016	–
	Grenå	*L. multiflorum*	DK22-M[a]	2017	–
	Haderslev	*L. perenne*	DK22-P[a]	2017	–
Greece	Arethousa	*L. rigidum*	GR9	2017	ACCase + ALS
	Drama	*L. rigidum*	GR20	2017	ACCase + ALS
	Kilkis	*L. rigidum*	GR24	2017	ACCase + ALS
	Drama	*L. rigidum*	GR30	2017	ACCase + ALS
	Leveon	*L. rigidum*	GR33[a]	2017	–
	Aliartos	*L. rigidum*	GR39[a]	2017	–
Italy	Ascoli Satriano	*L. rigidum*	IT533	2013	ACCase
	Chiarenta	*L. rigidum*	IT595	2016	ACCase + ALS
	Alessandria	*L. multiflorum*	IT609	2017	ACCase + ALS
	Caragna Piemonte	*L. multiflorum*	IT620	2017	ACCase + ALS
	Legnaro	*L. multiflorum*	IT204[a]		–

[a]*Reference populations.*

TABLE 2 | Herbicides used in the dose-response experiments.

Herbicide SoA (chemical family)	Commercial name (Manufacturer)	Active ingredient (a.i.)	Concentration a.i.	[b]Field dose (N) g a.i.ha^{-1}
ACCase (FOP[a])	Topik 240 (Syngenta)	Clodinafop-propargyl	240 g L^{-1}	60
		+ cloquintocet-mexyl (safener)	60 g L^{-1}	15
ACCase (DEN[a])	Axial pronto (Syngenta)	Pinoxaden	60 g L^{-1}	45
		+ cloquintocet-mexyl (safener)	15 g L^{-1}	10
ALS (SU[a])	Atlantis WG (Bayer CropScience)	Mesosulfuron-methyl +	30 g Kg^{-1}	15
		iodosulfuron-methyl sodium	6 g Kg^{-1}	3
		+ mefenpyr-diethyl (safener)	90 g Kg^{-1}	45

[a]*Aryloxyphenoxypropionate (FOP); cyclohexanedione (DEN); and sulfonylurea (SU).*
[b]*Recommended Italian field dose.*

extra sum-of-squares F test, available in GraphPad Prism 8, was performed to determine if the data of the two experiments could be pooled, i.e., if one curve adequately fitted both data set.

Resistance indexes (RIs) were calculated as the ratio between the ED_{50} (or GR_{50}) of the resistant and the susceptible population separately for each country. When the ED_{50} (or GR_{50}) could not be determined because plant survival (or fresh weight) was higher than 50% even at the highest herbicide doses, the maximum applied dose was used as a proxy for ED_{50}/GR_{50}.

Identification of Mutant *ALS* and *ACCase* Alleles

Five to ten ALS-resistant plants from the 12 *Lolium* populations were analyzed to detect the presence of mutant *ALS* and *ACCase* alleles. When no *ACCase* mutant alleles were detected another five ACCase-resistant plants were genotyped to confirm or reject the absence of mutant alleles indicating a putative non-target site resistance.

Total genomic DNA (gDNA) was extracted from 0.1 g leaf tissue using the CTAB method (Doyle and Doyle, 1987). A 1600 bp region of the CT domain of the plastidic *ACCase* gene was amplified by PCR on gDNA using the primers acclr9 and acclr6 (**Table 3**). The amplified region encompassed all the amino acid substitutions so far identified as conferring resistance. PCR amplifications were performed using GoTaq DNA Polymerase kit (Promega, United States) in a 25 μL mixture including 5 μL of 5 × Colorless GoTaq Flexi Buffer, dNTPs mix (0.2 mM each), $MgCl_2$ (3 mM), forward and reverse primers (0.4 μM each), 0.125 μL GoTaq DNA Polymerase, and 25 ng of gDNA. The thermocycler program was as follows: 95°C for 2 min; 45 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 2 min; 72°C for 5 min. PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co., Germany) following the manufacturer's instructions. Once purified, PCR products obtained from each plant were sequenced by BMR Genomics (Padova, Italy) using primers LOL_FOR and LOL_FOR_SEQ (**Table 3**).

Similarly, a 1719 bp fragment of the *ALS* gene was amplified from each DNA extracted with primers LOL_ALS_F and ALS_LOL_R reported in **Table 3**. PCR amplifications were performed using GoTaq DNA Polymerase kit (Promega, United States) in a 25 μL total volume containing 5 μL of 5× Colorless GoTaq Flexi Buffer, 5% of DMSO (1.25 μL), dNTPs mix (0.2 mM each), $MgCl_2$ (4 mM), forward and reverse primers (0.4 μM each), 0.125 μL GoTaq DNA Polymerase, and 25 ng of gDNA. The thermocycler program was as follows: 95°C for 2 min; 45 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 2 min; 72°C for 5 min. PCR products were purified as described for *ACCase* gene and sequenced by BMR Genomics using primers LOL_ALS_F and ALS_LOL_FS (**Table 3**).

RNA Extraction and q-PCR

Ten populations of *Lolium* spp. were chosen for this study, four susceptible populations and six resistant populations (DK29, DK90, GR24, GR30, IT533, and IT609) that had no (or sporadic) ALS and ACCase mutated plants. Resistant plants without mutant *ALS* and *ACCase* alleles were determined as described in the previous paragraph. All populations were sown in trays placed in a glasshouse at Flakkebjerg, Denmark. At BBCH 21, twenty plants from each population were separated into two individual plants (i.e., two clones) and transplanted into pots. A week later, one clone from each plant was sprayed with mesosulfuron-methyl + iodosulfuron-methyl sodium at 30 + 6 g ha^{-1} while the

TABLE 3 | List of primers used for the ALS and ACCase fragments amplification and sequencing.

Primer name	Sequence 5′-3′	Target
acclr9	ATGGTAGCCTGGATCTT GGACATG	Forward primer, *ACCase* CT domain amplification (Zhang and Powles, 2006)
acclr6	GGAAGTGTCATGCAATT CAGCAA	Forward reverse, *ACCase* CT domain amplification (Zhang and Powles, 2006)
LOL_FOR	CTGTCTGAAGAAGACTA TGGCCG	Sequencing *ACCase* gene
LOL_FOR_SEQ	GAGGTGGCTCAGCTAT GTTCCTG	Sequencing *ACCase* gene
LOL_ALS_F	CCGCAAGGGCGCCGACA TCCTCGT	Forward primer, *ALS* amplification
ALS_LOL_R	CGAAATCCTGCCATCAC CTTCCAT	Reverse primer, *ALS* amplification
ALS_LOL_FS	TCCATCACCAAGCACA ACTACCTC	Sequencing *ALS* gene

other clone remained non-treated. Plant responses to herbicide treatment was visually assessed 4 weeks after application. The treated clones were rated as susceptible or resistant while the non-treated clones were cut at the soil surface and frozen in liquid nitrogen immediately after harvest for subsequent gene expression analysis.

Total RNA was extracted from 50 mg of leaf material of three individual plants of the ten populations using RNeasy Plant Mini Kit (Qiagen, Stanford, CA, United States). The quality and concentration of RNA samples were determined as reported by Mahmood et al. (2016).

The qPCR reactions were performed with the GoTaq 1-step RT-qPCR System (Promega, Madison, WI, United States) using an Applied Bioscience ViiATM7 real-time PCR system with 384 wells (Thermo Fisher Scientific, Waltham, MA, United States). Reactions were performed in triplicates and a negative control consisting of reaction mix without template was also included for each primer. Briefly, 20 μL reaction mix included 10 μL GoTaq qPCR MasterMix 2×, 4 μL RNA template, 4 μL 0.5 pmol primers (1:1 mix of forward and reverse primers), 0.4 μL GoScript RT Mix 1-Step RT-qPCR 50x, 1.6 μL nuclease-free distilled water. Reaction conditions included 50°C for 5 min followed by 10 min incubation at 95°C, then 40 cycles of 95°C for 15 s and 60°C for 1 min. One internal control gene Rab GTPase (*RGTP*) and four herbicide metabolism genes *NMO*, *GST*, *CYP72A1,* and *CYP72A2* were chosen. Primer sequences are identical to those described by Gaines et al. (2014) and available in Mahmood et al. (2016).

Threshold-cycle (C_t) values were calculated for each reaction. Gene-specific PCR efficiency was used to calculate the expression of target genes in relation to the expression of internal reference gene. Equivalent slopes for target and internal control gene were observed in amplification plots. The ΔC_t value was calculated as follows: ΔC_t (target genes)=C_t (target gene) – C_t (reference gene), where C_t is the cycle number at which PCR product exceeded a set threshold. Relative transcript level (RTL) was calculated through = $1 \times 2^{-\Delta Ct}$ (Pfaffl, 2006). The significance levels for each gene expression were calculated for all pairwise comparisons through a single factor ANOVA followed by Tukey HSD (Honestly Significant Difference) test.

RESULTS

Dose-Response Bioassays

The extra sum-of-squares *F* test conducted on the survival and fresh weight data of each population to compare the dose-response curves obtained in the two experiments indicated that most of the curves were significantly different at $p < 0.05$. Therefore, it was not possible to estimate a common curve except for the survival data obtained with mesosulfuron + iodosulfuron (**Table 4**).

For mesosulfuron + iodosulfuron the estimated ED_{50} values of the three susceptible reference populations were similar, 55.9 g ha^{-1} (=1.68 g mesosulfuron + 0.33 g iodosulfuron) for population DK100 and 57 g ha^{-1} (=1.71 g mesosulfuron + 0.34 g iodosulfuron) for populations GR33 and IT204 (**Table 4**). The four Danish populations exhibited high level of resistance and even at the highest herbicide dose tested (120 + 24 g a.i. ha^{-1} of mesosulfuron + iodosulfuron) plant survival was higher than 50%. Similarly, the four Italian populations were highly resistant with RIs > 70 for three populations IT595, IT609, IT620 and RI = 20 for population IT533, while the Greek populations showed RIs ranging from 8 to >70.

TABLE 4 | Parameter estimates of the dose-response of Atlantis WG (field dose is 500 g ha^{-1} = 15 g mesosulfuron + 3 g iodosulfuron ha^{-1}) on *Lolium* spp. populations.

Population	Slope	ED_{50}	*SE*	*P*-value	RI
DK6		>4000			**>50**
DK29		>3000			**>37**
DK47		>4000			**>50**
DK90		>4000			**>50**
DK100*	−2.49	55.9	4.34	0.06	
GR9	−0.71	427	75.74	0.96	**8**
GR20	−1.24	602	64.16	0.43	**11**
GR24		>4000			**>70**
GR30	−1.17	3643	690.00	0.56	**64**
GR33*	−3.17	57	3.38	0.76	
IT533	−1.83	1521	150.00	0.06	**20**
IT595		>4000			**>70**
IT609		>4000			**>70**
IT620		>4000			**>70**
IT204*	−3.17	57	3.20	0.76	

*Herbicide dose that causes 50% reduction of the percentage of surviving plants (ED_{50}), relative standard error (SE), curve slope, P-value of lack-of-fit F-test and resistance index (RI) are shown. Data for spring and autumn experiment pooled. *Susceptible populations.*

The reference populations showed higher ED_{50} values with clodinafop, ranging from 29 to 55 g a.i. ha^{-1}, in the spring experiment than in the autumn experiment where the ED_{50} values ranged from 15 to 19 g a.i. ha^{-1}. In general, the RIs of all populations were higher in the autumn experiment compared to the spring experiment, however, the ranking of populations was consistent. The four Danish populations showed low to moderate resistance to clodinafop. The RIs for plant survival were between 2.3 and 6.8 in the spring experiment and between 3.4 and 7.3 in the autumn experiment. The RIs based on fresh weight recorded slight differences between both experiments with RIs ranged between 0.9–4.8 and 0.6–8.5 in the spring and autumn experiments, respectively (**Table 5**).

All four Greek populations were highly resistant to clodinafop with ED_{50} and GR_{50} values that corresponded to the higher doses tested (i.e., 480 g a.i. ha^{-1}) in both experiments. The resulted RIs were around 17 for both survival and fresh weight in the spring experiment and >32 or >135 in the autumn experiment. Similarly, all four Italian populations were highly resistant to clodinafop with higher values of RI recorded for the autumn experiment (**Table 5**).

Overall, the reference populations were completely controlled at half the recommended dose of pinoxaden (i.e., 22.5 g a.i. ha^{-1}). The ED_{50} values of the three reference populations ranged between 9 and 12 g a.i ha^{-1} in both experiments. Three Danish populations (DK6, DK29, and DK47) showed a slight shift in the

TABLE 5 | Dose-response experiments.

	Clodinafop (spring experiment)				Clodinafop (autumn experiment)			
POP	**ED_{50}**	**R.I.**	**GR_{50}**	**R.I.**	**ED_{50}**	**R.I.**	**GR_{50}**	**R.I.**
DK-6	238 (32.1)	**4.3**	206 (20.4)	**4.8**	122 (17.71)	**6.5**	2.9 (2.49)	**0.6**
DK-29	129 (15.4)	**2.3**	39 (10.2)	**0.9**	124 (16.02)	**6.6**	29.4 (7.23)	**6.6**
DK-47	147 (10.2)	**2.7**	188 (20.9)	**4.3**	63 (10.24)	**3.4**	5.4 (4.18)	**1.2**
Dk-90	372 (13.9)	**6.8**	171 (69.6)	**4.0**	136 (6.39)	**7.3**	38.1 (7.81)	**8.5**
DK-100*	55 (2.8)		43 (1.6)		18.8 (0.88)		4.5 (0.53)	
GR9	>480	**>17**	>480	**>16**	>480	**>32**	>480	**>135**
GR20	>480	**>17**	>480	**>16**	>480	**>32**	>480	**>135**
GR24	>480	**>17**	>480	**>16**	>480	**>32**	>480	**>135**
GR30	>480	**>17**	>480	**>16**	>480	**>32**	>480	**>135**
GR33*	29 (3.6)		31 (4.7)		14.8 (0.52)		3.6 (0.62)	
IT533	>480	**>11**	>480	**>16**	>480	**>27**	>480	**>104**
IT595	337 (61.1)	**8**	>480	**>16**	134 (27.93)	**8**	n.a.	
IT609	>480	**>11**	>480	**>16**	>480	**>27**	>480	**>104**
IT620	>480	**>11**	>480	**>16**	>480	**>27**	>480	**>104**
IT204*	42 (4.6)		30 (4.6)		17.5 (2.49)		4.6 (1.33)	

Clodinafop-propargyl doses (g a.i. ha^{-1}) causing 50% reduction in survival (ED_{50}) and fresh weight (GR_{50}) on Lolium spp. populations and resistance indexes (RIs). Standard errors are reported in brackets.
**Susceptible populations.*
n.a., not available because some pots were damaged.

susceptibility to pinoxaden with RIs ranging from 1.1 to 2.6 based on plant survival and 0.9 to 3.1 based on fresh weight in both experiments while the fourth population DK90 had a higher RI. Three out of four Greek populations (GR9, GR24, GR30) were highly resistant to pinoxaden, with RIs ranging from 6 to 12.6 and from 19 to 39, based on survival in the spring and autumn experiment, respectively. The fourth population (GR20) had a lower resistance level respect to the other Greek populations (RI = 3.1 in the spring experiment and 11.4 in the autumn experiment). Finally, three of the four Italian populations (IT533, IT609, and IT620) were moderately resistant to pinoxaden, with RIs ranging between 6.3 and 7 for plant survival and between 2 and 9.2 for fresh weight. The RI values based on fresh weight were similar for the autumn experiment while the RIs based on survival were higher (**Table 6**).

Mutant *ALS* and *ACCase* Alleles

Primers acclr9/acclr6 amplified a 1600 bp amplicon encompassing all codons of the *ACCase* gene known to confer resistance. Similarly, primers LOL_ALS_F/ALS_LOL_R amplified a 1719 bp amplicon encompassing all codons of the *ALS* gene conferring resistance. Both amplicons were sequenced for all the 83 plants (**Table 7**).

The sequencing of ACCase amplicons revealed that all the Greek and Italian populations had plants with a mutated *ACCase* allele (**Table 7**). Overall, in the Greek populations, six different *ACCase* mutant alleles were detected: Leu_{1781}, Cys_{2027}, Asn_{2041}, Val_{2041}, Gly_{2078}, and Arg_{2088}. In population GR30 only one type of mutant *ACCase* allele was detected (Leu_{1781}), in population GR9 two types (Leu_{1781} and Val_{2041}), in population GR20 four types (Cys_{2027}, Asn_{2041}, Gly_{2078}, and Arg_{2088}) as in population GR24 (Leu_{1781}, Cys_{2027}, Asn_{2041}, and Gly_{2078}). In addition, different mutant *ACCase* alleles were found in the same plant of population GR20.

Four different types of *ACCase* mutant alleles were found in plants from Italy. Populations IT595, IT609 and IT620 showed only one *ACCase* mutant allele each – Leu_{1781}, Asn_{2041} and Ala_{2096}, respectively. In population IT533, three mutant alleles were identified: Leu_{1781}, Gly_{2078}, and Ala_{2096}. In contrast, no mutant *ACCase* alleles were identified in any of the 20 plants analyzed from the Danish populations. It is noteworthy that several plants with no mutated *ACCase* allele were also identified within two Greek populations (GR24 and GR30) and in all the Italian populations (**Table 7**).

Six types of mutant *ALS* alleles (Ala_{197}, Gln_{197}, Leu_{197}, Ser_{197}, Thr_{197}, and Asn_{376}) were identified in the plants of the Greek populations. In populations GR20 and GR30 only one type of *ALS* mutant allele was detected (Ser_{197}), while in populations GR9 three types (Ser_{197}, Thr_{197}, and Asn_{376}) and in GR24 three types (Ala_{197}, Gln_{197}, and Leu_{197}) were identified. In three of the four Italian populations more than one mutant *ALS* allele was detected: Glu_{376} and Leu_{574} in IT595, Gln_{197} and Ser_{197} in IT609 and Gly_{122}, Leu_{197} and Val_{205} in IT620. The fourth population, IT533, had only the Glu_{376} allelic variant. Conversely, the plants of the Danish populations showed only one type of mutant *ALS* allele (Leu_{574}), and only in two populations (DK6 and DK47) (**Table 7**). The ALS and ACCase sequences presented can be found in a specific repository (Panozzo and Scarabel, 2020).

Gene Expression of Herbicide Metabolism Related Genes

Six of the resistant populations (DK29, DK90, GR24, GR30, IT533, and IT609) that had plants with no *ALS* and *ACCase*

TABLE 6 | Dose-response experiments.

	Pinoxaden (spring experiment)				Pinoxaden (autumn experiment)			
POP	**ED_{50}**	**R.I.**	**GR_{50}**	**R.I.**	**ED_{50}**	**R.I.**	**GR_{50}**	**R.I.**
DK-6	15 (0.2)	**1.3**	12 (0.7)	**1.6**	13.7 (0.2)	**1.1**	9.2 (0.2)	**0.9**
DK-29	28 (1.4)	**2.3**	16 (1.6)	**2.1**	32.9 (0.9)	**2.6**	31.5(1.3)	**3.1**
DK-47	14 (0.0)	**1.2**	15 (0.4)	**2.0**	19.7 (2.7)	**1.6**	11.6 (0.9)	**1.1**
DK-90	46 (5.3)	**3.9**	15 (2.2)	**1.9**	60.1 (4.8)	**4.7**	45.0 (3.0)	**4.4**
DK-100*	12 (0.0)		8 (1.2)		12.7 (0.1)		10.1 (0.1)	
GR9	52 (8.3)	**6.0**	58 (10.8)	**6.0**	177.3 (10.4)	**19.1**	>90	**>36**
GR20	27 (1.0)	**3.1**	30 (3.3)	**3.1**	105.8 (16.3)	**11.4**	52.9 (26.5)	**21.4**
GR24	94 (15.4)	**10.8**	47 (4.8)	**4.8**	300.5 (23.2)	**32.4**	>180	**>72**
GR30	109 (22.8)	**12.6**	73 (24.0)	**7.5**	>360	**>39**	>90	**>36**
GR33*	9 (0.0)		10 (1.8)		9.3 (0.6)		2.5 (0.2)	
IT533	85 (28.2)	**7.0**	85 (12.3)	**9.2**	211.3 (26.2)	**19.9**	36.8 (14.0)	**4.8**
IT595	20 (0.3)	**1.7**	21 (0.7)	**2.2**	18.4 (1.7)	**1.7**	25.9 (6.2)	**3.4**
IT609	84 (12.3)	**7.0**	30 (1.9)	**3.2**	68.5 (10.9)	**6.4**	51.9 (14.7)	**6.8**
IT620	77 (4.7)	**6.3**	18 (4.2)	**2.0**	130.0 (15.7)	**12.2**	24.8 (9.9)	**3.3**
IT204*	12 (0.2)		9 (1.1)		10.6 (0.0)		7.6 (0.1)	

Pinoxaden doses (g a.i. ha^{-1}) causing 50% reduction in survival (ED50) and fresh weight (GR50) on Lolium spp. populations and resistance indexes (RIs). Standard errors are reported in brackets.
**Susceptible populations.*

TABLE 7 | *ALS* and *ACCase* allelic variants identified in resistant *Lolium* spp. plants from Danish, Greek, and Italian populations compared to the susceptible plant.

	***ACCase* allelic variants**						***ALS* allelic variants**					**No mutant plant/no. analyzed plants**
Population	**1781**	**2027**	**2041**	**2078**	**2088**	**2096**	**122**	**197**	**205**	**376**	**574**	
DK6	–	–	–	–	–	–	–	–	–	–	Leu (5)	5/5
DK29	–	–	–	–	–	–	–	–	–	–	–	0/5
DK47	–	–	–	–	–	–	–	–	–	–	Leu (4)	4/5
DK90	–	–	–	–	–	–	–	–	–	–	–	0/5
GR9	Leu (3)	–	Val (1)	–	–	–	–	Ser (3)Thr (2)	–	Asn (1)	–	5/5
GR20	–	Cys (1)	Asn(3)	Gly (1)	Arg (1)	–	–	Ser (5)	–	–	–	5/5
GR24	Leu (1)	Cys (1)	Asn (1)	Gly (1)	–	–	–	Ala (1) Gln/Leu (1)	–	–	–	5/7
GR30	Leu (3)	–	–	–	–	–	–	Ser (1)	–	–	–	4/8
IT533	Leu (2)	–	–	Gly (1)	–	Ala (1)	–	–	–	Glu (1)	–	5/10
IT595	Leu (5)	–	–	–	–	–	–	–	–	Glu (4)	Leu (4)	9/10
IT609	–	–	Asn (1)	–	–	–	–	Gln (1)Ser (1)	–	–	–	3/10
IT620	–	–	–	–	–	Ala (3)	Gly (2)	Leu(1)	Val(1)	–	–	5/8
[a]IT204	Ile	Trp	Ile	Asp	Cys	Gly	Ala	Pro	Ala	Asp	Trp	

For each codon, the amino acid substitution identified is indicated and the number of plants carrying the mutation is reported in bracket. Dashes indicate amino acids identical to those in susceptible plant from IT-204[a].

mutant alleles were further studied to determine the expression patterns of four genes, *NMO*, *GST*, *CYP72A1*, *CYP72A2* known to be involved in herbicide metabolism. Four susceptible *Lolium* spp. populations were also examined (DK39, DK100, DK22M and DK22P).

For all four genes, significant differences between plants of the same population were observed. For example, the expression of *NMO* in plant DK100-1 was around 5.5 fold higher than in plants 2 and 3 (**Figure 1A**) and the expression in plant IT609-3 was 9 and 3.7 fold higher respect to plant 1 and 2, respectively. These differences were observed within all populations, except population IT533, implying that in general *Lolium* populations are heterogeneous for the gene expression of one or more herbicide metabolism genes. No significant differences in the expression of NMO were observed between susceptible and resistant populations (**Figure 1A**).

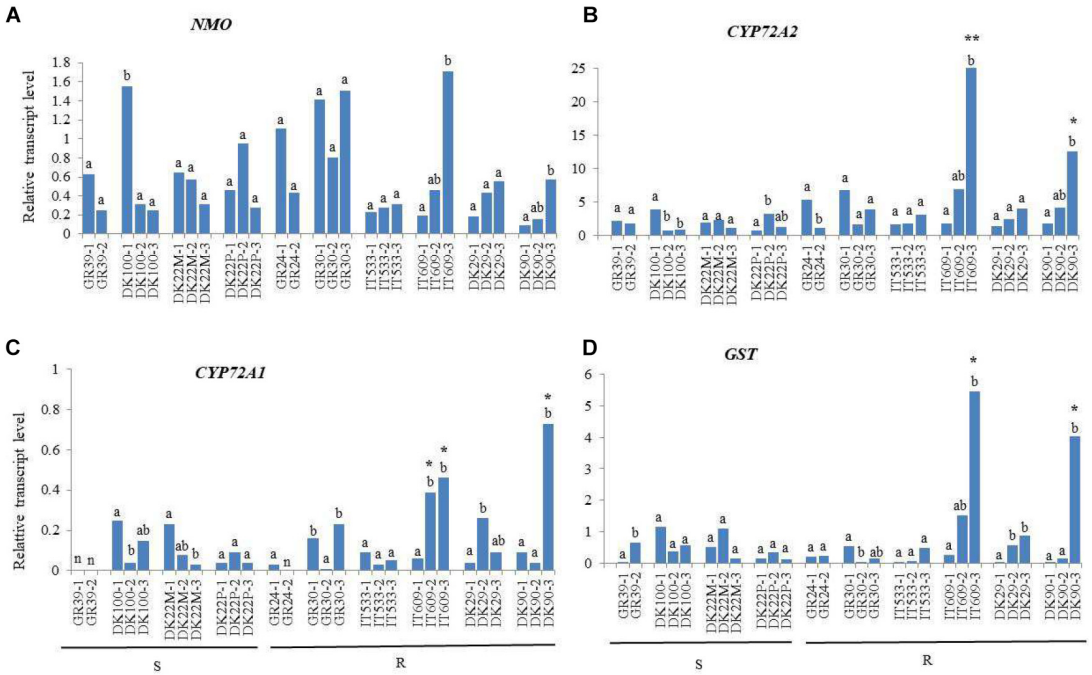

FIGURE 1 | Relative transcript levels of *NMO* **(A)**, *CYP72A2* **(B)**, *CYP72A1* **(C)**, and *GST* **(D)** in leaves of *Lolium* spp. plants resistant (R) or susceptible (S) to mesosulfuron-methyl + iodosulfuron-methyl relative to the internal reference gene *RGTP*. Significant differences between plants of the same population are indicated with different letters. Asterisks denote significant differences between R plant compared to all S plants tested (**) or compared to plants of only one S population (*). *n* = samples with Ct values below no template control.

Among the genes studied, *CYP72A2* was expressed at the highest level in the resistant plant (IT609-3) with a relative expression value of 24.99 while the lowest expression was found in the susceptible plant DK22P-1 with RTL value of 0.72. The expression of *CYP72A2* was significantly higher in plant IT609-3 compared to all the susceptible plants analyzed, and in the resistant plant DK90-3 compared to plants from the susceptible population DK22M, from 5 to 11-fold up-regulated. Other resistant plants, GR24-1, GR30-1 and IT609-2 exhibited higher expression respect to few susceptible plants. For example, *CYP72A2* was 9.4 fold higher expressed in plant GR30-1 respect to the susceptible plant DK22P-1 but only 1.8 fold higher respect to plant DK100-1 (**Figure 1B**).

The *CYP72A1* gene was expressed at a relatively low level as indicated by its generally low RTL values ranging from 0.03 to 0.73 However, a significant ($p < 0.001$) increased expression was found in plants IT609-2, IT609-3 and DK90-3. *CYP72A1* was 8-fold up-regulated in the resistant plant IT609-3 compared on average to the susceptible plants of population DK22P and 7-fold up-regulated in the resistant plant IT609-2 compared to the same susceptible population. Plant DK90-3 exhibited the highest relative expression value for *CYP72A1* (0.73) and the gene was 13-fold up-regulated compared on average to the susceptible plants of population DK22P and was also significantly up-regulated respect to plants DK100-2, DK100-3, DK22M-2 and DK22M-3 with 18, 5, 9 and 24-fold up-regulation, respectively (**Figure 1C**).

The *GST* gene showed significantly ($p < 0.001$) up-regulation in plant IT609-3 and DK90-3, whereas among the susceptible plants, population DK22P showed very low relative expression (on average RTL value of 0.19) as well as plant GR39-1 (RTL = 0.04) and DK22M-3 (RTL = 0.15). *GST* was 30-fold up-regulated in the resistant plant IT609-3 compared on average to the susceptible plants of population DK22P and was also significantly up-regulated compared to some plants of other susceptible populations, GR39-1, DK22M-1, DK22-M3 and DK100-2 with 136, 11, 36 and 14-fold up-regulation, respectively. Similarly, *GST* was 22-fold up-regulated in plant DK90-3 in comparison with the susceptible plants of population DK22P and was also significantly up-regulated compared to some plants of other susceptible populations, GR39-1, DK22-M3 and DK100-2 with 101, 27 and 11-fold up-regulation, respectively (**Figure 1D**). *GST* showed the highest differences in expression between susceptible and resistant populations respect to the other genes studied.

DISCUSSION

Occurrence of Multi-Resistant *Lolium* spp.

This study confirmed the occurrence of *Lolium* spp. populations multi-resistant to ALS and ACCase inhibitors in Denmark, Italy and Greece and highlighted differences in the pattern and level of resistance among countries and populations. TSR appears to be responsible for the resistance status of Greek populations, and for most of the Italians. Conversely, resistance in Danish populations

is totally endowed by NTSR mechanism in case of ACCase inhibitors and by both NTSR and TSR in case of ALS inhibitors.

While all populations were highly resistant to mesosulfuron-methyl + iodosulfuron-methyl, the susceptibility to both ACCase inhibitors, clodinafop-propargyl and pinoxaden was generally much lower in the Danish populations compared to the Italian and Greek ones. A low resistance level has often been associated to the presence of non-target-site resistance mechanisms, however, in some cases the level of resistance can be higher due to the build-up over time of different NTSR mechanisms (Cocker et al., 2001; Kaundun, 2014). Among the investigated populations, the level of resistance to pinoxaden varied among populations in each country and among countries. These differences in the pattern and level of resistance could be related to different cropping practices and herbicides used in the three countries (Llewellyn and Powles, 2001; Owen et al., 2014). The herbicide pinoxaden is not authorized in Denmark while it is frequently used as a post-emergence application in Italian and Greek winter cereals fields. This supports the higher resistance indexes generally observed in the Italian and Greek populations. However, even if pinoxaden is not used, a low resistance level to pinoxaden has been detected in one Danish population. Instead, a low but clear resistance to clodinafop was found. This type of resistant phenotype as well as the absence of mutated *ACCase* alleles strongly suggests the presence of a NTSR mechanism.

Diversity of *ACCase* and *ALS* Alleles Endowing Resistance

ACCase variant alleles endowing resistance were present in all the Greek and Italian populations studied. Overall, six different types of *ACCase* variant alleles were detected in the Greek populations: Leu_{1781}, Cys_{2027}, Asn_{2041}, Val_{2041}, Gly_{2078}, and Arg_{2088} and four in the Italian populations: Leu_{1781}, Asn_{2041}, Gly_{2078}, and Ala_{2096}. Depending on the population considered, one to four different *ACCase* alleles were observed in the same population. Similar results were reported by Yu et al. (2007) who found different *ACCase* mutant alleles in Italian *Lolium* populations resistant to clethodim and showed that homozygous plants having Leu_{1781}, Gly_{2078} or Arg_{2088} were also resistant to other ACCase inhibitors including clodinafop and pinoxaden. In a subsequent study conducted on Italian *Lolium* spp. populations resistant to pinoxaden, the same *ACCase* variant alleles as detected in our work were found, except for Cys_{2027} (Scarabel et al., 2011). In contrast, in all the four Danish populations investigated no ACCase variant alleles were detected indicating that target site resistance is not present.

The analyses of the *ALS* gene indicated that only one *ALS* variant allele (Leu_{574}) endowing resistance to mesosulfuron-methyl + iodosulfuron-methyl was present in two Danish populations while in the other two, no *ALS* variants were detected. Conversely, in the Greek and Italian populations different *ALS* variant alleles were found, six (Ala_{197}, Gln_{197}, Leu_{197}, Ser_{197}, Thr_{197}, Asn_{376}) in the Greek populations and seven in the Italian ones (Gly_{122}, Gln_{197}, Leu_{197}, Ser_{197}, Val_{205}, Glu_{376}, Leu_{574}) and diversity of *ALS* alleles was detected in some populations. The Italian populations showed amino acid substitutions at five different codons of the *ALS* gene. This is in accordance with the study of Yu et al. (2008), who, in a single Australian population, identified six different mutations in the *ALS* gene endowing resistance to chlorsulfuron. This is not surprising as *Lolium* spp. are obligate cross-pollinated species and therefore pollination among resistant plants from neighboring fields can occur within 3 km distance increasing the genetic heterogeneity of the *Lolium* plants (Busi et al., 2008). In the Greek populations, the majority of amino acid substitutions endowing resistance was observed at codon Pro-197 and this is in accordance with previous findings (Kaloumenos et al., 2012; Anthimidou et al., 2020). This substitution was frequently reported in numerous grass weeds and it usually confers resistance only to sulfonylureas (such as mesosulfuron-methyl + iodosulfuron-methyl) (Yu and Powles, 2014). Instead, the Leu_{574} *ALS* allele, present only in two Danish populations, endows high resistance to all chemical group of ALS inhibitors (Heap, 2020). The variability in the ALS mutations detected in the three countries confirms the differences observed in the cross-resistance pattern.

Some plants in two Greek populations (GR24 and GR30) and in all the Italian populations showed no amino acid substitutions endowing resistance to ALS and ACCase. This suggests that a different resistance mechanism (i.e., NTS) is likely present.

Metabolism-Based Resistance in *Lolium* spp.

Enhanced metabolism-based resistance is considered the prevalent resistance mechanism in grass weeds and its complex genetic control (polygenic control) involves the regulation of specific genes (Délye, 2013). The cytochromes P450 belong to a supergene family and are involved in all the pathways of plant secondary metabolism (Werck-Reichhart and Feyereisen, 2000). They play a major role in the phase I of metabolic herbicide detoxification and in the coordination with the GST enzymes involved in phase II of herbicide detoxification (Cocker et al., 2001; Yuan et al., 2007). GSTs include a large, complex gene family in plants that catalyze the conjugation to various substrates and oxidatively produced compounds to reduced glutathione, which facilitates their metabolism and sequestration (Dalton et al., 2009). Nitronate monooxygenase is a flavin-dependent enzyme that oxidizes anionic alkyl nitronates. It is active on a broad range of substrates containing primary and secondary nitro groups and its involvement in detoxification of propionate-3-nitronate was reported in yeast and bacteria (Gadda and Francis, 2010). In *A. thaliana*, NMO was found to be associated with detoxification of the allelochemical benzoxazolin (Baerson et al., 2005).

The high expression of *CYPs* and *GST* is expected to enhance herbicide degradation in resistant plants (Délye, 2013). The expression level of both *CYPs* studied (*CYP72A2* and *CYP72A1*) varied broadly from plant to plant and the same was observed for the gene *GST*. This variability of expression between plants implies that the populations are generally heterogeneous for the gene expression of one or more herbicide metabolism genes. Similar findings were reported by Duhoux and Délye (2013) who

reported that the expression of five *CYP* genes, both constitutive and herbicide-induced, varied broadly from plant to plant in a French *Lolium* spp. population. Despite the variability among plants, our data showed that the expression levels of *CYP72A1*, *CYP72A2,* and *GST* were significantly higher in resistant plants in population IT609 and DK90 compared to the expression level of the susceptible plants. This proves that an enhanced herbicide degradation is present in these plants and highlights the evolution of metabolic based resistance in these *Lolium* populations. *CYP72A* gene was found to be involved in metabolic resistance to diclofop in *L. rigidum* (Gaines et al., 2014). Moreover, enhanced *GST* expression was shown to determine an acceleration in the herbicide degradation in a clodinafop-resistant *L. rigidum* population (Gaines et al., 2014) and in Danish *L. multiflorum* population (Mahmood et al., 2016).

No clear distinction in *NMO* expression was observed between resistant and susceptible plants. This gene was identified as candidate gene by RNAseq transcriptome analysis involved in metabolism-based diclofop resistance in *L. rigidum* (Gaines et al., 2014). *NMO* was found to be two-fold up-regulated in glufosinate-resistant *Amaranthus palmeri* respect to susceptible plants, both constitutively and herbicide-induced (Salas-Perez et al., 2018).

The no clear distinction of expression in the genes studied that are specific to the resistant plant may be related to the fact that additional genes not studied here are involved in the herbicide metabolization. Two cytochrome P450 genes (*CYP81A12* and *CYP81A21*) were found to be overexpressed and associated with resistance to ALS inhibitors in *Echinochloa phyllopogon* (Iwakami et al., 2014). Recently, these genes were found to be involved in the detoxification of ACCase inhibitors in multiple herbicide resistant *E. phyllopogon* (Iwakami et al., 2019). Because of the high number and variability of CYPs and GSTs, plants have the potential to overcome the herbicide treatment trough a concerted action of several genes. It was demonstrated that the genetic control of P450 metabolism-based resistance mechanism in *Lolium rigidum* is governed by a set of genes that varied among plants, even in a given population (Busi et al., 2011).

In conclusion, in the present plant material, IT609-3 and DK90-3 exhibited high expression of *GST, CYP72A1,* and *CYP72A2* genes constitutively, implying that herbicide resistance for these populations could be attributed to an elevated level of herbicide metabolism.

Coevolution of Resistance Mechanisms

In the last decade there has been an increase in multi-resistant *Lolium* spp. populations in Europe (Heap, 2020). This work highlights the presence of a wide variety of multi-resistant *Lolium* spp. populations in Denmark, Greece, and Italy and provides strong evidence that two different resistance mechanisms (TSR and NTSR metabolism-based resistance) co-evolved.

A diversity of mutant *ALS* and *ACCase* alleles were detected among plants of the Greek and Italian populations indicating a high population heterogeneity. In the Danish populations, only one type of mutant *ALS* allele was found. However, in both situations, evolution of target-site resistance is suggested to be the result of a strong selection pressure imposed by the herbicides used. The frequent use of the ALS inhibitor, mesosulfuron + iodosulfuron and lower use of ACCase inhibitors in Danish cereals fields compared with the common herbicide usages in Italy and Greece may explain this difference.

The high variability among plants observed in the expression profile of the four genes involved in the metabolism of mesosulfuron + iodosulfuron indicates that even if plants are subjected to the same herbicide selective pressure, a different weed response to the chemical agent can occur. It is likely that since EMR is a polygenic trait, the evolution process over time allows accumulation of various favorable traits able to increase the survival of the plant and the transmission to the next generation.

The presence of different resistance mechanisms increases the complexity of resistant *Lolium* spp. management in cereal fields. From a practical point of view, TSR determines resistance to herbicides with the same SoA, while metabolism-based resistance can not only endow resistance to the selecting herbicides but also confers cross-resistance to herbicides having different SoA. Numerous genes can be involved in metabolism-based resistance and, according to their regulation, they can confer resistance to different chemicals (Gaines et al., 2014). Therefore, EMR mechanism observed in some *Lolium* populations investigated in this study is of concern because the simple rotation over the years of ALS and ACCase inhibiting herbicides will not be effective, as pointed out by Collavo et al. (2013).

CONCLUSION

This work displays the heterogeneity in the pattern and level of resistance to ALS and ACCase inhibitors in Danish, Greek, and Italian *Lolium* spp. populations and demonstrates the presence of target-site resistance and coexistence of metabolic based herbicide resistance mechanism in populations from Denmark and Italy. The potential of evolution of enhanced metabolism-based resistance is an important threat to consider for improving practices against resistance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of this study, shared plant materials, contributed to give comments, revised the manuscript, and read and approved the final manuscript. DL, LS, MK, SM, and SP performed the experiments and analyzed the data. LS wrote the first draft of the manuscript.

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ORIGINAL RESEARCH
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Predominance of Metabolic Resistance in a Six-Way-Resistant Palmer Amaranth (*Amaranthus palmeri*) Population

*Chandrima Shyam†, Ednaldo A. Borgato†, Dallas E. Peterson, Johanna Anita Dille and Mithila Jugulam**

Department of Agronomy, Kansas State University, Manhattan, KS, United States

Evolution of multiple herbicide resistance in Palmer amaranth across the United States is a serious challenge for its management. Recently, a Palmer amaranth population (KCTR; Kansas Conservation Tillage Resistant) from a long-term conservation tillage research project in Kansas, United States, was found uncontrolled by several commonly used herbicides. Importantly, this field did not have a history of repeated use of some of the herbicides for which the KCTR Palmer amaranth population showed lack of control. The objectives of this study were to confirm the evolution of multiple resistances and determine possible mechanism(s) of resistance in KCTR Palmer amaranth plants. In response to post-emergence application, 28–100% of KCTR Palmer amaranth survived field recommended rates of 2,4-D, ALS-, PS II-, EPSPS-, PPO-, HPPD-inhibitor herbicides, or tank- or pre-mixture of PS II- and HPPD-inhibitor herbicides, confirming evolution of six-way resistance in this Palmer amaranth population. However, this population was found susceptible to the PS I- and glutamine synthetase inhibitor herbicides. Chlorsulfuron-, imazethapyr-, and atrazine-resistant plants did not show any previously reported mutation in *ALS* and *psbA* genes, the target sites of these herbicides, respectively. However, the survivors of glyphosate treatment showed amplification of *EPSPS* gene (up to 88 copies). The KCTR plants pretreated with cytochrome P450 or GST inhibitors along with atrazine, 2,4-D, lactofen, or mesotrione had significantly less biomass accumulation than those treated with herbicides alone. Plants treated with P450 inhibitor followed by imazethapyr showed moderate reduction of biomass in KCTR which was statistically similar to a susceptible Palmer amaranth population treated with imazethapyr. These results suggest predominance of metabolic resistance possibly mediated by cytochrome P450 and GST enzyme activity that may have predisposed the KCTR Palmer amaranth population to evolve resistance to multiple herbicides. This is the first report of evolution of six-way resistance in a single Palmer amaranth population. Appropriate management strategies, including integration of cultural, and mechanical, and herbicide mixtures, are warranted to control such Palmer amaranth populations.

Keywords: metabolism, inhibitor assays, *EPSPS* amplification, cytochrome P450 monooxygenases, glutathione S-transferases

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Lena Ulber,
Julius Kühn-Institut—Braunschweig, Germany

***Correspondence:**
Mithila Jugulam
mithila@ksu.edu

† These authors have contributed equally to this work

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INTRODUCTION

Palmer amaranth (*Amaranthus palmeri* S. Watson) is a top-ranked troublesome weed in the United States (Van Wychen, 2019). It is a summer annual C_4 eudicot species, with a fast growth rate and ability to accumulate biomass (Horak and Loughin, 2000; Sellers et al., 2003). These biological attributes make Palmer amaranth a highly competitive species and if uncontrolled can contribute to drastic yield losses of up to 91% in corn, 79% in soybean, 59% in cotton, and 50% in sorghum (Massinga et al., 2001; Morgan et al., 2001; Bensch et al., 2003; Moore et al., 2004). Palmer amaranth has currently evolved resistance to eight herbicide sites of action (SOAs) in the United States, including 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS-), acetolactate synthase- (ALS-), photosystem II- (PS II-), 4-hydroxyphenylpyruvate dioxygenase- (HPPD-), protoporphyrinogen oxidase- (PPO-), long-chain fatty acid- (LCFA-), microtubule-inhibitor herbicides, and synthetic auxins (Heap, 2020). Previously, resistance to up to five SOAs, i.e., ALS-, EPSPS-, PS II-, HPPD-inhibitor herbicides, and synthetic auxins, was reported in different populations of Palmer amaranth from Kansas (Nakka et al., 2017a,b,c; Kumar et al., 2019; Chaudhari et al., 2020).

Weed resistance to herbicides, especially multiple-herbicide resistance, poses a serious threat to global food production. Both target-site (TSR) and non-target-site (NTSR) resistance mechanisms have been found to confer resistance to herbicides in Palmer amaranth. TSR mechanisms involving alterations in the target site of the herbicide such as amino acid substitutions or deletions and increased copy number and/or expression of the target gene have been reported in this species (Gaines et al., 2010; Salas et al., 2016; Nakka et al., 2017b,c). Mutations in the gene encoding herbicide-targeted enzymes can reduce herbicide-binding activity leading to resistance. Specifically, in Palmer amaranth single amino acid substitutions, i.e., A122S or A122T, P197S or P197A, T574L, and S653A were reported to confer resistance to ALS-inhibitors in weed species (Küpper et al., 2017; Nakka et al., 2017c; Singh et al., 2019). Palmer amaranth populations resistant to PPO-inhibitor herbicides were found to have the amino acid substitutions R128M/G (also referred as R98), and G399A, as well as a codon (glycine) deletion at the position 210 (Δ210) in *PPX2* gene coding for the target enzyme of PPO-inhibitor herbicides (Salas et al., 2016; Giacomini et al., 2017; Salas-Perez et al., 2017; Varanasi et al., 2017; Rangani et al., 2019). Another commonly identified TSR mechanism in Palmer amaranth is the amplification of the *EPSPS* gene, the molecular target of glyphosate (Gaines et al., 2010; Chahal et al., 2017; Molin et al., 2017; Singh et al., 2018). Furthermore, the amplified *EPSPS* gene copies are present in the form of extrachromosomal circular DNA (eccDNA), with an autonomous replication site, and are randomly inherited during cell division (Koo et al., 2018; Molin et al., 2020). After the first case of glyphosate-resistant Palmer amaranth from Georgia, United States in 2005, it has rapidly spread throughout the United States becoming a serious challenge for agriculture. Apart from *EPSPS* amplification, a mutation in the *EPSPS* gene leading to P102S substitution has also been reported in Palmer amaranth (Kaundun et al., 2019).

In contrast to TSR, NTSR mechanisms do not directly alter the target site but reduce the amount of active herbicide reaching the target site due to either reduced absorption, translocation, or increased metabolism of the herbicide. Specifically, in metabolic resistance, the active herbicide is broken down into non-toxic forms before it reaches the target site, thus reducing its efficacy. Reduced absorption and translocation imparting glyphosate resistance in a Palmer amaranth population from Argentina have been reported (Palma-Bautista et al., 2019). However, enhanced herbicide detoxification *via* cytochrome P450 monooxygenase (P450s) and glutathione *S*-transferase (GSTs) activity is the most common NTSR mechanism reported in ALS-, PS II-, HPPD-, and PPO-inhibitor-resistant Palmer amaranth (Nakka et al., 2017a,b,c; Varanasi et al., 2018). Additionally, GSTs were found to be involved in *S*-metolachlor resistance in this species (Brabham et al., 2019).

P450s and GSTs are groups of enzymes important to the catalysis of several xenobiotic compounds in living organisms, including herbicides in many crops and weeds (Dixon et al., 2010; Pandian et al., 2020). Importantly, metabolic resistance can confer cross- or multiple-resistance (Jugulam and Shyam, 2019). The P450s from CYP81A subfamily have been shown to impart cross-resistance to several herbicide classes including ALS-, ACCase-, PS II-, phytoene desaturase- (PDS-), PPO-, HPPD-, and 1-deoxy-D-xylulose 5-phosphate synthase- (DOPX-) inhibitor herbicides in late watergrass (*Echinochloa phyllopogon*; Dimaano et al., 2020). Likewise, a phi (F) class GST, *AmGSTF1*, was shown to detoxify multiple herbicides in blackgrass (*Alopecurus myosuroides*; Cummins et al., 2013).

We previously reported a Palmer amaranth population from Kansas (KSR) with resistance to ALS-, PS II-, and HPPD-inhibitor herbicides (Nakka et al., 2017a,b,c). In this population, predominance of metabolic resistance *via* P450 or GST activity to all the above herbicides was found (Nakka et al., 2017a,b,c). Importantly, the field where the KSR population was collected had a history of use of ALS- and PS II-, but not HPPD-inhibitor herbicides, validating the implications of metabolic resistance in bestowing cross-resistance to herbicides that were not previously used (Nakka et al., 2017b; Nandula et al., 2019). More recently, a three-way resistant Palmer amaranth population (HMR) was documented in Kansas with resistance to ALS-, PS II-, and EPSPS-inhibitor herbicides (Chaudhari et al., 2020). Several mutations in the *ALS* gene, such as P197S, P197T, P197A, and P197A, or T574L were documented in HMR. *EPSPS* amplification was observed in this population with copies ranging from 50 to 140. No mutation was observed in *psbA* gene fragments of this population implying the involvement of NTSR imparting atrazine resistance.

In 2018, a Palmer amaranth population (KCTR) from a long-term conservation tillage experimental field (Department of Agronomy, Kansas State University) grown with continuous sorghum for over 45 years was found to survive post-emergence (POST) application of several commonly used herbicides, including 2,4-D and atrazine. These herbicides have been routinely used in this field to control broadleaf weeds. Since Palmer amaranth has evolved resistance to

eight SOAs (Heap, 2020) and there was a predominance of metabolic resistance in other Palmer amaranth populations in Kansas, the KCTR Palmer amaranth was used in this research to confirm and characterize resistance. The objectives of this research were to (1) confirm evolution of resistance in KCTR to several POST herbicides and (2) determine if TSR or NTSR mechanisms confer resistance to multiple herbicides.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Ten KCTR plants that survived 2,4-D treatment (560 g ae ha^{-1}) and showed active growth after herbicide application were collected (summer 2018) and brought to the weed science greenhouse at Kansas State University. These plants were transplanted into pots (15 × 10 × 15 cm) for seed production. Seeds produced from several female plants were harvested, cleaned, and pooled to evaluate their response to multiple herbicides. Susceptible populations including one from Kansas (KSS) and one from Mississippi (MSS) were used for comparisons. All experiments were conducted in the above greenhouse maintained at 30/23°C ± 2°C (day/night temperatures) with 60% ± 10% relative humidity, and 14/10 h day/night photoperiod supplemented with 250 μmol m^{-2} s^{-1} illumination provided by sodium vapor lamps.

Response of KCTR to Post-emergence (POST) Herbicide Application

Seeds of KCTR, KSS, and MSS populations were germinated in plastic trays (21 × 6 × 4 cm) filled with a commercial potting mixture (Pro-Mix® premium potting mix, Premier Tech Home and Garden Inc., Ontario, CA). After emergence, seedlings were individually transplanted into pots (6 × 6 × 6.5 cm) and grown under greenhouse conditions as previously described. This experiment was performed in a completely randomized design with 18 treatments including field recommended rates of 17 POST herbicides (**Table 1**) and non-treated control. Twenty-five replicates were maintained for each treatment, and the experiment was repeated. In total, 50 plants (from two runs) from each of the KCTR and KSS or MSS population were treated with these POST herbicides (**Table 1**). Adjuvants were included following manufacturer instructions (**Table 1**). Herbicides were applied using a bench-track sprayer (Generation III, DeVries Manufacturing, RR1 Box 184, Hollandale, MN) equipped with a flat-fan nozzle tip (8002 Teejet, Spraying Systems Co., Wheaton, IL) calibrated to deliver a spray volume proportional to 187 L ha^{-1} at 4.77 km h^{-1}. Plant survival (alive or dead) was assessed at 2 weeks after treatment (WAT) with PPO-inhibitor herbicides; 3 WAT for glyphosate, ALS-, HPPD-, and PS II-inhibitor herbicides; and 4 WAT for 2,4-D. The percent survival (**Table 2**) was calculated by dividing the number of plants that survived herbicide by total number of plants treated, considering both experimental runs.

Assessment of TSR Mechanisms in KCTR Palmer Amaranth

DNA Isolation and Sequence Comparisons of *ALS* and *psbA* Genes in KCTR, KSS, and MSS Palmer Amaranth

Approximately 100 mg of young leaf tissue was collected from the survivors of chlorsulfuron- (n = 3; n: number of plants) and imazethapyr- (n = 16) and atrazine (n = 22) treated KCTR, and non-treated KSS (n = 1) and MSS (n = 5) plants for DNA isolation. After collection, leaf tissue was homogenized using a prechilled mortar and pestle with liquid nitrogen. Total genomic DNA (gDNA) was extracted using a Genomic DNA Extraction kit (Thermo Fisher Scientific, Waltham, MA). DNA was quantified using a nanodrop (Nanodrop 1000, Thermo Fisher Scientific), and quality was verified using 0.8% agarose gel electrophoresis prior to further steps. Polymerase chain reactions (PCR) were performed using T100™ Thermal Cycler (Bio-Rad Inc., Hercules, CA) to amplify the *ALS* and *psbA* genes, the target site of these herbicides. Individual reactions included 80 ng of DNA, 2 μL of each forward and reverse primers (5 μM), 10 μL of PCR master mix (GoTaq Green Master Mix, 2×, Promega PCR Master Mix, Fisher Scientific Company, Ontario, Canada), and molecular-grade water totalizing 25 μL per reaction. Primer sets used to amplify the *ALS* and *psbA* genes were designed by Mengistu et al. (2005) and Whaley et al. (2007), respectively, and have previously been used in our lab (Nakka et al., 2017a,c). For *ALS* gene amplification, the following PCR conditions were maintained: 95°C for 5 min and 35 × 95°C for 1 min, 57°C for 30 s, 2 min at 72°C, and 10 min at 72°C. PCR conditions consisted of 95°C for 5 min for initial denaturation and 35 cycles of 95°C for 30 s for denaturation, 55°C for 30 s for annealing, 72°C for 45 s for extension, and 10 min at 72°C for final extension for *psbA*. PCR products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific) following the manufacturer instructions and sent for Sanger sequencing at the Genewiz facilities (Genewiz Inc., South Plainfield, NJ). For *ALS* sequencing, along with the forward and reverse primers used for PCR, an internal primer (ALS_F2-5′-AACAGCCCATTAAATTGGGTG-3′) was used. The *psbA* gene was sequenced with the same forward primer used for PCR. Multiple alignments of *ALS* and *psbA* gene sequences of KCTR, KSS, and MSS sequences were performed using Geneious Prime® software (Biomatters Inc., Newark, NJ).

Relative *EPSPS* Genomic Copy Number Estimation

Leaf tissue of KCTR plants (n = 13) that survived glyphosate treatment were collected to estimate the *EPSPS* copy number relative to *β-Tubulin* using a real-time quantitative PCR (RT-qPCR). DNA extraction was performed as described above, and qPCR was performed using a StepOnePlus™ real-time detection system (Applied Biosystems, Waltham, MA). Each qPCR reaction consisted of 8 μL of PowerUp™ SYBR™ Green master mix (Applied Biosystems), 2 μL each of forward and reverse primers (5 μM) (Gaines et al., 2010), and 2 μL of gDNA (20 ng μL^{-1}) with 14 μL total. *β-Tubulin* was used as endogenous control as described by Godar et al. (2015). Individual reactions

TABLE 1 | POST-emergence herbicide treatments used for screening KCTR, MSS, and KSS Palmer amaranth populations.

WSSA group	SOA	Herbicides	Dose[a] (g ha^{-1})	Product	Manufacturer
2	ALS inhibitors	Chlorsulfuron	18	Glean® [b]	Corteva Agriscience, Willington, DE
		Thifensulfuron	36	Harmony SG[b]	Corteva Agriscience
		Imazamox	35	Beyond[b]	BASF Corp., Research Triangle Park, NC
		Imazethapyr	36	Pursuit[b]	BASF Corp.
4	Synthetic auxins	2,4-D	560	2,4-D 4L Amine	Winfield Solutions, LLC, St. Paul, MN
5	PS II inhibitors	Atrazine	2,240	Aatrex 4L[c]	Syngenta Crop Protection, LLC., Greensboro, NC
		Metribuzin	140	Sencor 75[c]	Bayer Crop Science, Centreway Green Way, NC
9	EPSPS inhibitors	Glyphosate	840	Roundup WeatherMAX[d]	Bayer Crop Science
10	Glutamine synthetase inhibitors	Glufosinate	655	Liberty 280 SL[d]	BASF Corporation
14	PPO inhibitors	Lactofen	175	Cobra[c]	Valent U.S.A. Corp., Walnut Creek, CA
		Fomesafen	264	Flexstar[b]	Syngenta Crop Protection
22	PS I inhibitors	Paraquat	560	Gramaxone SL 2.0[c]	Syngenta Crop Protection
27	HPPD inhibitors	Mesotrione	105	Callisto[c]	Syngenta Crop Protection
		Tembotrione	92	Laudis[d,e]	Bayer Crop Science
5 + 27	PS II + HPPD inhibitors	Atrazine + mesotrione	1,120 + 105	Aatrex 4L + Calisto[c]	Syngenta Crop Protection
		Bromoxynil + pyrasulfotole	288	Huskie[b,d]	Bayer Crop Science

[a] *Field recommended rate to control Palmer amaranth.*
[b] *Nonionic surfactant at 0.25% v/v.*
[c] *Crop oil concentrate at 1% v/v.*
[d] *Ammonium sulfate (34%) at 2% v/v; methylated seed oil at 1% v/v.*
[e] *Methylated seed oil at 1% v/v.*

were performed with DNA collected from thirteen different KCTR plants that survived glyphosate application as biological replicates, with three technical replicates per DNA sample for both target and endogenous control genes. This experiment was repeated, and data was combined. To determine the specificity of the qPCR products, a melt curve profile was included following the thermal cycling. The *EPSPS* copy number in KCTR was estimated using the formula for fold induction ($2^{-\Delta\Delta Ct}$) (Pfaffl, 2001) relative to the reference sample, i.e., glyphosate-susceptible KSS or MSS plants, in each run. The mean *EPSPS* copy number of KCTR plants along with susceptible KSS and MSS were plotted along with standard error of mean calculated from two experimental runs.

TABLE 2 | Percent survival of KCTR Palmer amaranth population to different post-emergence herbicides.

WSSA group	SOA	Herbicides[a]	Survival[b] (%)
2	ALS inhibitors	Chlorsulfuron	34
		Thifensulfuron	60
		Imazamox	70
		Imazethapyr	60
4	Synthetic auxins	2,4-D	84
5	PS II inhibitors	Atrazine	100
		Metribuzin	36
9	EPSPS inhibitors	Glyphosate	28
10	Glutamine synthetase inhibitors	Glufosinate	0
14	PPO inhibitors	Lactofen	84
		Fomesafen	29
22	PS I inhibitors	Paraquat	0
27	HPPD inhibitors	Mesotrione	90
		Tembotrione	84
5 + 27	PS II inhibitors + HPPD inhibitors	Atrazine + mesotrione	42
		Bromoxynil + pyrasulfotole	98

[a] *A total of 50 plants from each population (KCTR and KSS or MSS) were sprayed with each herbicide.*
[b] *KSS and MSS were controlled (≥ 95%) with all the herbicides.*

POST Herbicide Efficacy With Cytochrome P450 and GST Inhibitors for Assessing Presence of NTSR Mechanisms in KCTR Palmer Amaranth

Whole-plant bioassays were conducted to investigate the presence of P450- and GST-mediated metabolic resistance to the herbicides (imazethapyr, atrazine, 2,4-D, mesotrione, and lactofen) for which Palmer amaranth and common waterhemp (*Amaranthus tuberculatus*) has been reported to have evolved such resistance. This experiment was performed under a completely randomized design with a factorial arrangement and was repeated. Treatments included combination of Palmer amaranth populations (resistant and susceptible) and chemical treatments (described below), with at least 8 replicates. Resistant (KCTR) and susceptible (KSS or MSS; based on availability of seeds) Palmer amaranth populations were compared. Chemical treatments included a) herbicide only, b) enzymatic inhibitor only (either P450 and/or GST inhibitor; depending on the herbicide), c) combination of enzymatic inhibitor with a

herbicide, and d) a non-treated control. Herbicide doses included application of field recommended rates of imazethapyr, mesotrione, atrazine, lactofen, and 2,4-D (**Table 1**). Based on published literature, both P450 and GST inhibitors were included to evaluate the metabolic resistance to lactofen for a total of six treatments, i.e., (a) lactofen only, (b) malathion only, (c) NBD-Cl only, (d) combination of malathion with lactofen, (e) combination of NBD-Cl with lactofen, and (f) non-treated control. The treatments were applied using a bench-track sprayer with appropriate adjuvants as described before. Malathion (Spectracide®, Spectrum Group, St. Louis, MO), a P450 inhibitor, was applied at 2,000 g ai ha^{-1} at least 30 min prior to herbicide application and also soil-applied (5 mM, 50 mL solution pot^{-1}) at 48 h after herbicide application as described by Ma et al. (2013) in combinations with or without imazethapyr, lactofen, and mesotrione. Our preliminary study (unpublished) to test the effect of malathion at 1,500 and 2,000 g ai ha^{-1} on 2,4-D efficacy in 2,4-D-resistant common waterhemp from Nebraska indicated that malathion at 1,500 g ai ha^{-1} was sufficient to increase susceptibility of common waterhemp to 2,4-D. Palmer amaranth is a close relative of common waterhemp; therefore, for assessing 2,4-D metabolism in KCTR, malathion was used at 1,500 g ai ha^{-1}, followed by soil application as described above. The GST inhibitor, NBD-Cl (Sigma Aldrich, St. Louis, MO), was applied at 270 g ai ha^{-1}, 48 h before atrazine or lactofen applications (Ma et al., 2013). Experiments were performed twice. Aboveground biomass was harvested at 2 WAT for lactofen; 3 WAT for glyphosate, imazethapyr, mesotrione, and atrazine; and 4 WAT for 2,4-D, oven-dried at 65°C for 72 h, and quantified. Biomass data were converted to percent dry weight relative to the non-treated control for statistical analysis.

Levene's test ($\alpha = 0.05$) was conducted to compare runs, and if no significance was identified, relative dry weight data were combined. Normality of residuals and homoscedasticity of variances were verified prior to ANOVA, and relative dry weight data was square root-transformed. Data were subsequently fitted to a linear mixed effect model using the "*nlme*" package and the function "*lme*" available in R (version 4.0.3, R Core Team, 2020) with the R-Studio 9.4 interface (R Studio, PBC, Boston, MA) considering Palmer amaranth populations and chemical treatments as fixed effect and experimental runs as a random effect. If interaction across populations and treatments was significant, the means were separated using Tukey's test using the "*multcompview*" and "*lsmeans*" packages at $\alpha = 0.05$. Data for adjusted means were back-transformed to calculate percent reduction in biomass with application of inhibitors in comparison to herbicide alone. These results were plotted using "*ggplot2*" package for graphical visualizations.

RESULTS

Percent Survival of KCTR and KSS or MSS in Response to POST Herbicide Treatment

Percent survival of KCTR plants to different herbicides was highly variable indicating the considerable genetic variability KCTR Palmer amaranth population. Overall, >28% of KCTR plants survived field-recommended rates of all herbicides tested, except paraquat and glufosinate, for which this population was found to be susceptible (**Table 2**). Following 2,4-D treatment, KSS plants (∼10%) were recorded with green tissue, weak twisted stem, and dried meristem (**Figure 1**). However, since no growth as well as presence of dried meristem was observed following 2,4-D treatment, these plants were considered as dead. Overall, > 95% control of either KSS or MSS plants was recorded with all herbicide treatments (**Table 2**). The lowest percent survival of KCTR plants was found for glyphosate (28%). In response to several ALS-inhibitor herbicides, the KCTR plants showed variation in % survival as follows: 34% for chlorsulfuron, 60% for thifensulfuron and imazethapyr, and 70% for imazamox suggesting that KCTR has evolved resistance to both sulfonylureas and imidazolines.

In response to PS II-inhibitor (e.g., atrazine and metribuzin) application, all KCTR plants survived atrazine but only 36% survived treatment with metribuzin, confirming the evolution of resistance to PS II-inhibitor herbicides (**Table 2**). Eighty-four and 90% of KCTR plants survived mesotrione and tembotrione treatments, respectively, suggesting prevalence of resistance to HPPD-inhibitor herbicides (**Table 2**). Nonetheless, 42% of KCTR plants survived the tank mixture of atrazine and mesotrione (**Table 2**). Additionally, 98% of KCTR plants also survived the commercial premix of bromoxynil (PS II-inhibitor) + pyrasulfotole (HPPD-inhibitor), one of the widely used POST herbicides for Palmer amaranth control in grain sorghum production. In response to PPO-inhibitor applications, 29 and 84% of KCTR plants survived treatment with fomesafen and lactofen, respectively, confirming evolved resistance to PPO-inhibitor herbicides (**Table 2**). Also, 84% of KCTR plants survived 2,4-D treatment at the field recommended rate.

Assessment of TSR Mechanisms in KCTR Palmer Amaranth

Nucleotide sequence alignment of the *ALS* gene of the KCTR, KSS, and MSS plants showed lack of the four previously reported mutations (Nakka et al., 2017c; Küpper et al., 2017; Singh et al., 2019; Chaudhari et al., 2020) known to confer resistance ALS-inhibitor herbicides in Palmer amaranth (**Figure 2**). Even though some nucleotide polymorphisms were detected, none of them were consistent among the resistant plants or resulted in amino acid substitution (**Figure 2**). No nucleotide polymorphisms were detected in the *psbA* sequence of KCTR plants (**Figure 3**). Our qPCR results indicated that glyphosate-resistant KCTR plants had increased number of *EPSPS* copies, ranging from 20 to 88, compared to KSS or MSS (**Figure 4**).

Assessment of NTSR Mechanisms in KCTR Palmer Amaranth

Malathion treatment alone did not significantly impact biomass accumulation in either KCTR, KSS or MSS plants (**Figures 5A,C–E**). Contrary to that, imazethapyr treatment resulted in significantly ($p < 0.0001$) lower biomass accumulation in MSS plants (9%) compared to KCTR plants (23%) (**Figure 5A**).

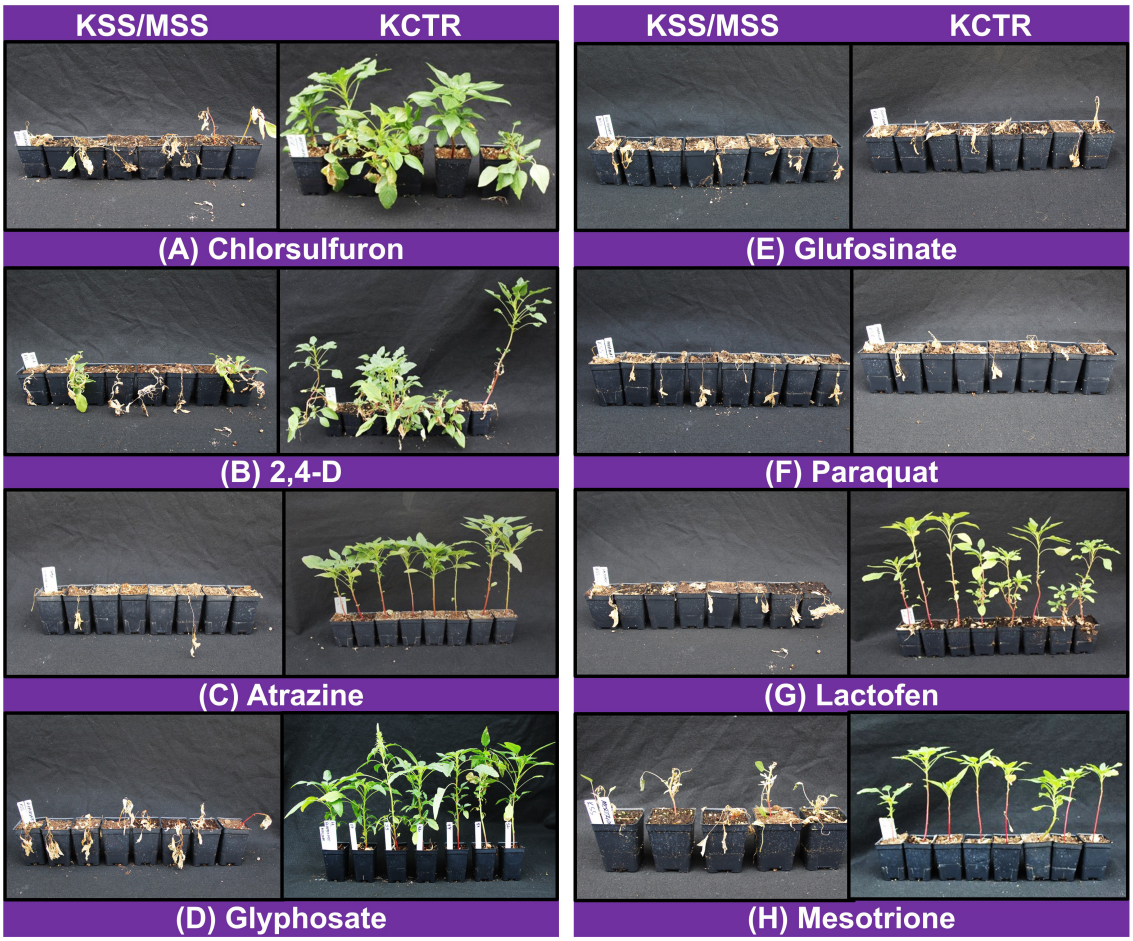

FIGURE 1 | Response of susceptible (MSS or KSS) and resistant (KCTR) Palmer amaranth to chlorsulfuron **(A)**, 2,4-D **(B)**, atrazine **(C)**, glyphosate **(D)**, glufosinate **(E)**, paraquat **(F)**, lactofen **(G)**, and mesotrione **(H)** application 2–4 weeks after treatment.

Treatment of malathion with imazethapyr did not reduce the relative biomass accumulation of KCTR plants compared to KCTR plants treated with imazethapyr alone (**Figure 5A**). Interestingly, there was no significant difference between KCTR plants treated with malathion with imazethapyr (15%) in comparison to MSS plants treated with either imazethapyr alone (9%) or malathion with imazethapyr (8%; **Figure 5A**).

Similar to malathion, NBD-Cl treatment alone did not significantly affect biomass accumulation in either KCTR, KSS, or MSS plants (**Figures 5B,E**). Atrazine treatment at the field-recommended rate resulted in 58% relative biomass accumulation in KCTR, whereas MSS plants had 3% biomass accumulation (**Figure 5B**). This is similar to the results of the herbicide screening experiment (**Table 2**). Treatment of NBD-Cl 48 h prior to atrazine resulted in only 33% relative biomass accumulation in KCTR plants which was significantly lower than atrazine only treatment (58%; $p < 0.0001$). Such effect of malathion was not observed with pretreatment of NBD-Cl in MSS plants (**Figure 5B**).

Highly variable biomass accumulation was observed in KCTR plants treated with 2,4-D; however, relative biomass accumulation in 2,4-D-treated KCTR plants (81%) was higher than 2,4-D-treated KSS plants (21%) (**Figure 5C**). Treatment of malathion with 2,4-D resulted in statistically lower biomass accumulation (45%) than only 2,4-D-treated KCTR plants ($p = 0.0005$). However, such impact of malathion was not observed in KSS plants (**Figure 5C**).

Mesotrione treatment resulted in significantly lower biomass accumulation in KCTR plants (65%) compared to KSS plants (8%) (**Figure 5D**). Nonetheless, treatment of malathion with mesotrione resulted in lower biomass accumulation in KCTR plants (31%) than mesotrione treatment alone ($p < 0.0001$) (**Figure 5D**). Contrary to that, malathion- and mesotrione-treated KSS plants (8%) showed no difference in biomass accumulation compared to mesotrione only treatment (**Figure 5D**).

Lactofen-only treatment resulted in significantly lower ($p = 0.0418$) relative biomass accumulation in KCTR plants (26%) compared to KSS plants (11%). Even with significant biomass reduction in the resistant population, several plants survived this herbicide application (**Table 2** and **Figure 5E**). The KSS Palmer amaranth was susceptible to all treatments containing lactofen

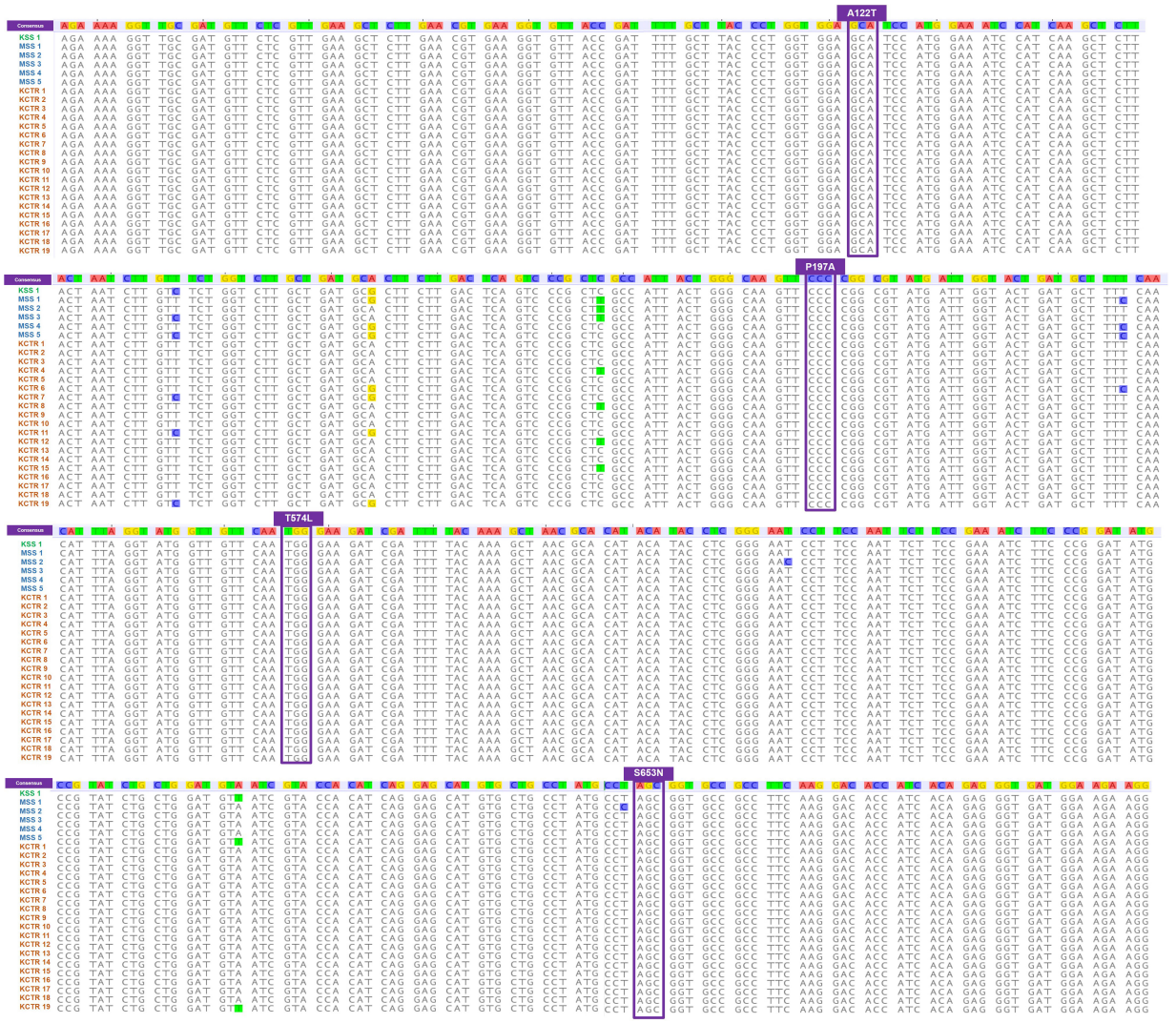

FIGURE 2 | Nucleotide sequence alignment of *ALS* gene fragment from susceptible (MSS and KSS) and chlorsulfuron (KCTR 1–3)- and imazethapyr (KCTR 4–19)-resistant Palmer amaranth. Nucleotide polymorphisms were observed across KCTR, KSS, and MSS Palmer amaranth, but no amino acid substitutions were found.

(**Table 2** and **Figure 5E**). Treatment of NBD-Cl fb lactofen did not result in increased sensitivity of KCTR (**Figure 5E**). However, combination of malathion with lactofen significantly reduced biomass accumulation in KCTR plants (8%), compared to the lactofen-only treatment. Such impact of malathion was not observed in KSS plants (**Figure 5E**).

DISCUSSION

Palmer amaranth is a dioecious species with prolific seed production that has already evolved resistance to eight herbicide SOAs (Heap, 2020). In this research, we report for the first time the evolution of six-way resistance in a single Palmer amaranth population, i.e., KCTR with predominance of metabolic resistance mechanisms. Previously Palmer amaranth populations with resistance to three and five SOAs have been reported (Nakka et al., 2017a,b,c; Kumar et al., 2019; Chaudhari et al., 2020). Similar to our findings, the six-way resistant common waterhemp population was also documented in Missouri (Shergill et al., 2018). Our results confirm the evolution of resistance to ALS-, PS II-, HPPD-, PPO-, EPSPS-inhibitor herbicides, and synthetic auxins in the KCTR population (**Table 2**). KCTR plants survived (28–100%) application of these herbicides, while satisfactory control (0% survival) was achieved only with PS I- (e.g., paraquat) and glutamine synthetase inhibitor herbicides (e.g., glufosinate) (**Table 2**). Such a wide range of survival for different SOAs indicates that KCTR is likely to be not genetically homogeneous, and possibly there is ongoing segregation for resistance to these SOAs, especially ALS- and EPSPS-inhibitor herbicides (**Table 2**). This is not unusual considering the amount of genetic variability offered by

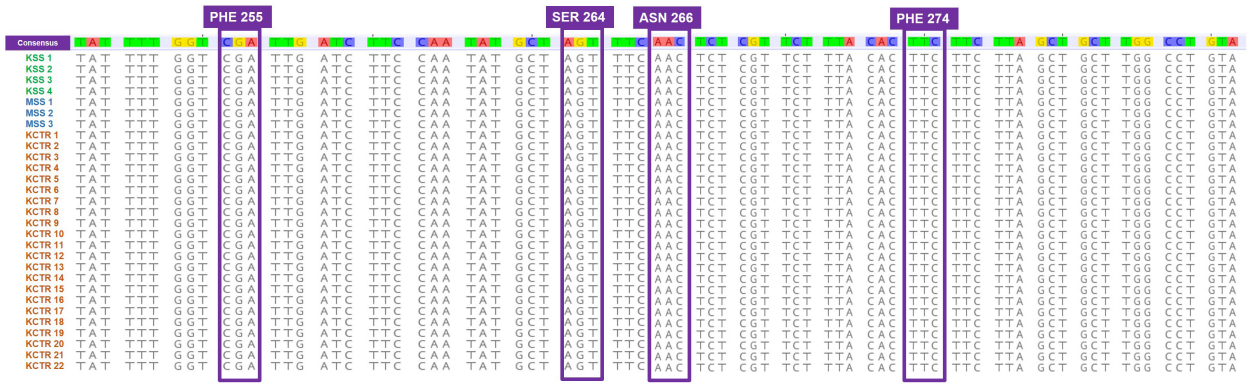

FIGURE 3 | Nucleotide sequence alignment of *psbA* gene fragments from atrazine-susceptible (MSS and KSS) and -resistant (KCTR) Palmer amaranth individuals. No nucleotide polymorphisms were observed among KCTR, KSS, and MSS Palmer amaranth individuals.

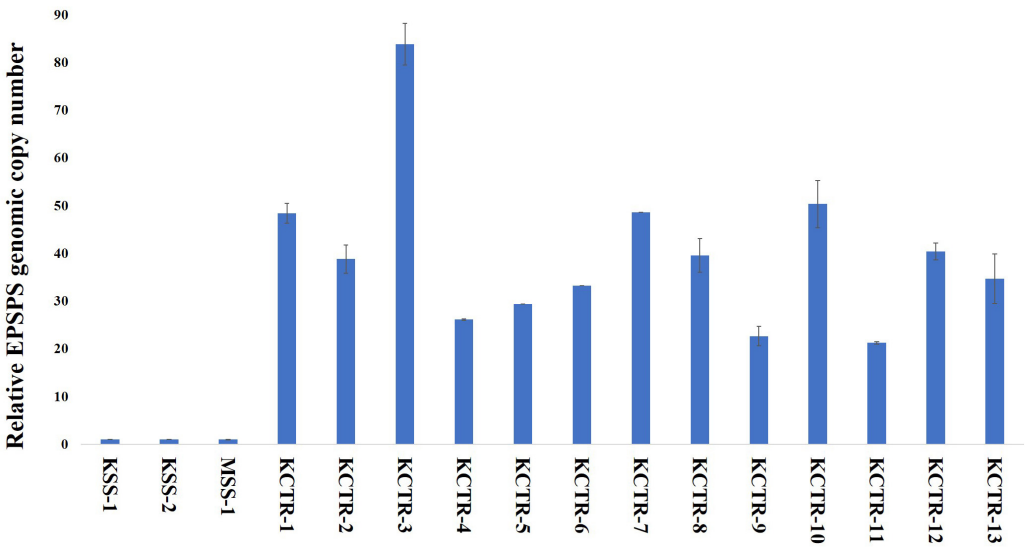

FIGURE 4 | *EPSPS* genomic copy number in glyphosate-susceptible (MSS and KSS) and glyphosate-resistant (KCTR) Palmer amaranth plants relative to the susceptible plants. KCTR plants were treated with the recommended field rate of glyphosate. Error bars represent the standard error from the mean (2 runs and in each run 3 technical replicates). The qPCR data was normalized using β-tubulin as the reference gene.

an outcrossing weed species like Palmer amaranth. Improved control of KCTR plants (58%) was observed with a tank mixture of atrazine (half of the recommended rate: 1,120 g ai ha^{-1}) and mesotrione (recommended rate: 105 g ai ha^{-1}) (**Table 1**) compared to atrazine alone (0%) or mesotrione alone (16%). However, some plants were not effectively controlled by this tank mixture. Such improvement in control can be attributed to the synergism often observed with tank mixing PS II- and HPPD-inhibitor herbicides (Abendroth et al., 2006; Chahal et al., 2019a). Additionally, the commercial pre-mixture of bromoxynil (PS II-inhibitor) and pyrasulfotole (HPPD-inhibitor) performed poorly in comparison to a tank mix of atrazine and mesotrione.

Although mutations in herbicide target genes conferring resistance are rare evolutionary events, mutations in the *ALS* gene at eight positions (four positions in Palmer amaranth) were found to confer resistance to several classes of ALS inhibitors in weeds (Tranel and Wright, 2002; Yu and Powles, 2014; Heap, 2020). Several amino acid substitutions at the *ALS* gene have been documented in Palmer amaranth populations from Kansas (Nakka et al., 2017c; Chaudhari et al., 2020). Interestingly, upon sequencing the whole *ALS* gene, the KCTR plants showed no presence of any of these substitutions (**Figure 2**). Malathion has been shown to increase the metabolic half-life of herbicides by inhibiting P450-dependent metabolism (Kreuz and Fonné-Pfister, 1992). Therefore, malathion treatment was done prior and after imazethapyr to test involvement of P450-based detoxification mechanisms in KCTR. Even though imazethapyr treatment was not significantly different from the combination of malathion with imazethapyr in reducing KCTR biomass, malathion with imazethapyr-treated KCTR plants produced the same level of biomass as imazethapyr-treated MSS plants (**Figure 5A**). This indicated moderate

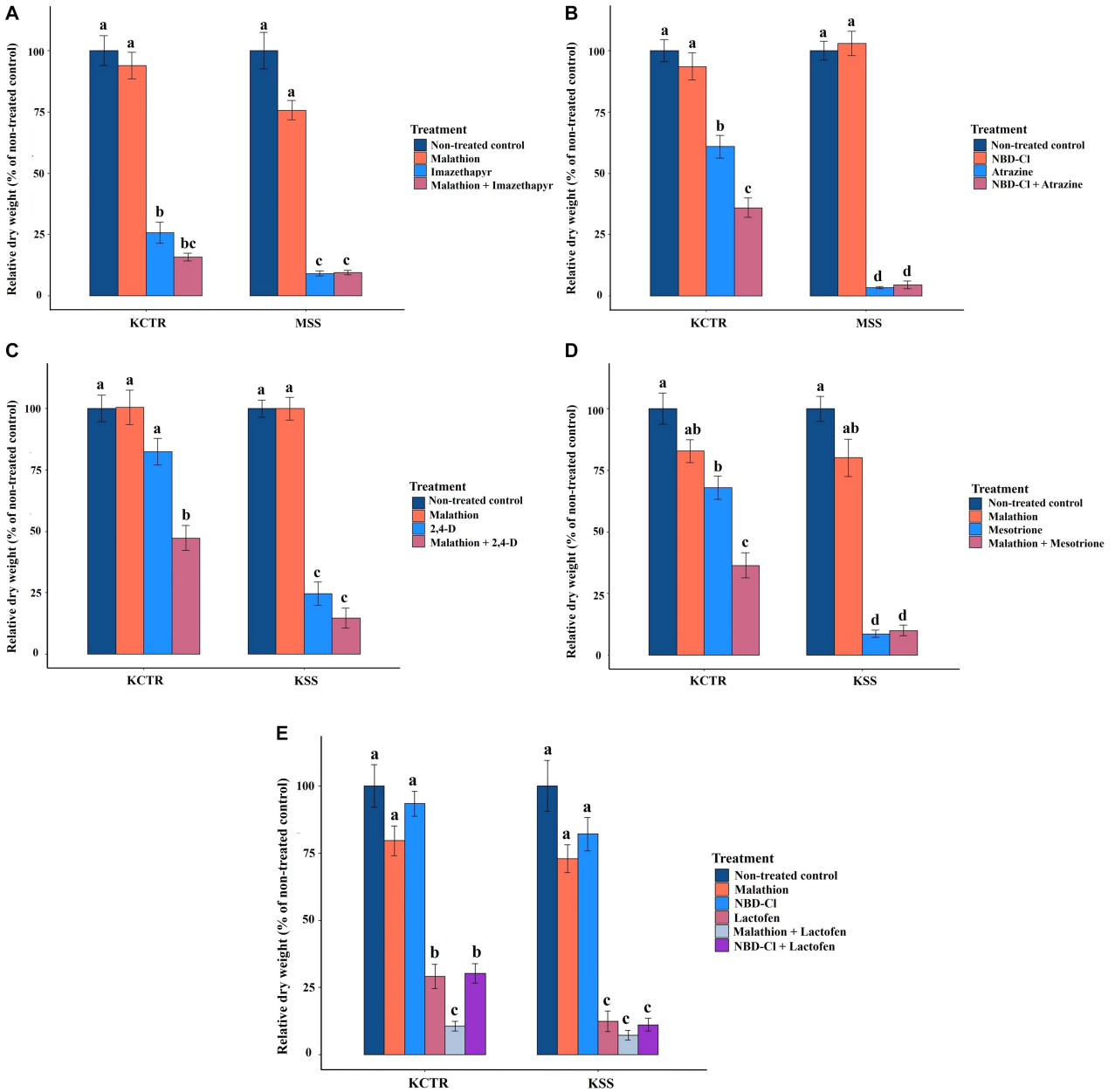

FIGURE 5 | Effect of P450 and GST inhibitors on efficacy of imazethapyr **(A)**, atrazine **(B)**, 2,4-D **(C)**, mesotrione **(D)**, and lactofen **(E)** in susceptible (KSS or MSS) and resistant (KCTR) Palmer amaranth populations. Error bars represent standard error of the mean. Letters represent significant differences identified by separation of means using Tukey (5%).

reduction of biomass in KCTR with malathion treatment. Moreover, since only 60% of KCTR plants were found to be resistant to imazethapyr (**Table 2**), it is possible that sensitive plants in KCTR may have contributed to the lack of differences between imazethapyr only and combination of malathion with imazethapyr treatment. Different P450 inhibitors have been observed to have varying levels of synergistic effect in resistant weeds depending on the herbicide as well as P450 isozyme. For instance, Preston et al. (1996) observed piperonyl butoxide (PBO; another P450 inhibitor) inhibiting chlorotoluron and simazine resistance in rigid ryegrass (*Lolium rigidum*), but malathion failed to do so. Similarly, Oliveira et al. (2018) observed that malathion improved the efficacy of tembotrione but not of mesotrione in HPPD-inhibitor-resistant common waterhemp from Nebraska. Therefore, it is possible that different P450s, which are not completely inhibited by malathion, are involved in imparting resistance to imazethapyr in KCTR.

All KCTR plants survived atrazine application (**Table 2**), and the lack of any known mutations in the *psbA* suggests

a NTSR mechanism to atrazine as reported in other Palmer amaranth populations (Nakka et al., 2017b; Chaudhari et al., 2020). A V219L mutation in the *psbA* gene was found in atrazine-resistant Powell amaranth (*Amaranthus powellii*; Dumont et al., 2016) but not in common waterhemp or other *Amaranthus* species closely related to Palmer amaranth (Ma et al., 2013; Shergill et al., 2018; Chahal et al., 2019b). NBD-Cl derivatives have been shown to give strong GST inhibition in human tumor cells and are termed as suicide inhibitors of GSTs (Ricci et al., 2005). Therefore, NBD-Cl treatment prior to atrazine application was given to KCTR plants to determine the involvement of GSTs in imparting resistance. This treatment significantly reduced biomass accumulation in KCTR plants indicating the involvement of GST enzymes in metabolizing atrazine. Previously, pretreatment with NBD-Cl has reversed atrazine resistance in common waterhemp (Ma et al., 2013; Shergill et al., 2018). Metabolic resistance to atrazine *via* glutathione conjugation mediated by GST activity has been reported in several Palmer amaranth and common waterhemp populations in the United States Midwest (Ma et al., 2013; Shergill et al., 2018; Chahal et al., 2019b). KCTR plants are also resistant to metribuzin, another PS II inhibitor. Metribuzin resistance mediated by enhanced metabolism was reported in wild radish (*Raphanus raphanistrum*; Lu et al., 2019) and rigid ryegrass (Ma et al., 2020).

Treatment of malathion with application of 2,4-D, mesotrione, or lactofen significantly reduced biomass accumulation in KCTR plants (**Figures 5C,E**), suggesting P450 enzymes-mediated detoxification of these herbicides in KCTR plants. Previously, malathion-induced reversal of weed resistance to 2,4-D (Shergill et al., 2018), carfentrazone, and fomesafen (Varanasi et al., 2018; Obenland et al., 2019), and mesotrione (Ma et al., 2013) has been reported. However, NBD-Cl failed to impact biomass accumulation in KCTR when applied prior to lactofen treatment. This indicates that certain P450 enzymes and not GSTs, or potentially specific GSTs not inhibited by NBD-Cl, may be involved in imparting resistance to lactofen in KCTR plants. In contrast to our findings, in the PPO-inhibitor-resistant Palmer amaranth population from Arkansas, the use of NBD-Cl caused the reversal of resistance to fomesafen (Varanasi et al., 2018).

EPSPS amplification (up to 88 copies) was found to contribute to glyphosate resistance in KCTR plants. Gaines et al. (2011) have shown that > 30 *EPSPS* copies are needed to withstand the field rate of glyphosate application (840 g ai ha^{-1}) in Palmer amaranth. Despite a low percentage (28%; **Table 2**) of glyphosate survivors in this population, lack of fitness penalty associated with this resistance mechanism and the obligate outcrossing nature of Palmer amaranth can lead to the rapid spread and transfer to other susceptible populations *via* pollen (Sosnoskie et al., 2012; Giacomini et al., 2014).

Based on previous research in our laboratory, the coexistence of both TSR and NTSR for the same herbicide can occur in a single population or individual plant of Palmer amaranth (Nakka et al., 2017a,b; Chaudhari et al., 2020). Research is in progress to determine if such a scenario is present in KCTR as well. Future investigations focused on identifying specific P450s and GSTs involved in herbicide detoxification in this population will also be investigated. It is important to understand what predisposes a population to develop metabolic resistance to several SOAs, including those with no history of use and absence of selection pressure.

Because pre-emergence (PRE) herbicide treatments are regarded as one of the best strategies to manage herbicide resistance in weeds (Norsworthy et al., 2012), information on response of KCTR to PRE herbicides can help in formulating viable options to manage this population. Therefore, experiments are in progress to investigate the response of KCTR to several soil-applied residual PRE herbicides (e.g., atrazine, mesotrione, flumioxazin, and *S*-metolachlor) commonly used to control germinating and emerging seedlings of Palmer amaranth. Previously, there has been a fitness penalty associated with multiple-herbicide resistance in weed species such as rigid ryegrass (Vila-Aiub et al., 2005). Studies will also be conducted to assess whether any fitness penalty is present in this population as a result of evolution of six-way herbicide resistance, which can help in understanding the spread of resistance traits to other populations.

Evolution of resistance to six herbicide SOAs in the KCTR population leaves very few POST herbicide options for its control. Moreover, such accumulation of resistance traits in a single Palmer amaranth population poses serious questions on the effectiveness of stacked resistance traits in crops, such as 2,4-D + glyphosate + glufosinate or dicamba + glyphosate resistance in corn and beans. Considering the lack of introduction of new SOAs, it is crucial to conserve currently available SOAs to effectively manage weeds. Growers should be encouraged to adopt integrated weed management techniques to reduce selection pressure by herbicides and discourage further selection of the evolution of multiple resistance.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication (the accession number for ALS and psbA sequences are MW361337 and MW361342).

AUTHOR CONTRIBUTIONS

DP identified KCTR Palmer amaranth population in the conservation tillage field. MJ and DP conceived research hypothesis, methodology, led, and supervised the research. CS and EB conducted the research, collected, and analyzed data (equal contribution). JD critically reviewed the manuscript and provided valuable comments. All authors read, edited, and approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-target-Site Resistance in *Lolium* spp. Globally: A Review

*Andréia K. Suzukawa[1], Lucas K. Bobadilla[2], Carol Mallory-Smith[1] and Caio A. C. G. Brunharo[1]**

[1] Department of Crop and Soil Science, Oregon State University, Corvallis, OR, United States, [2] Department of Crop Sciences, University of Illinois, Urbana, IL, United States

The *Lolium* genus encompasses many species that colonize a variety of disturbed and non-disturbed environments. *Lolium perenne* L. spp. *perenne*, *L. perenne* L. spp. *multiflorum*, and *L. rigidum* are of particular interest to weed scientists because of their ability to thrive in agricultural and non-agricultural areas. Herbicides are the main tool to control these weeds; however, *Lolium* spp. populations have evolved multiple- and cross-resistance to at least 14 herbicide mechanisms of action in more than 21 countries, with reports of multiple herbicide resistance to at least seven mechanisms of action in a single population. In this review, we summarize what is currently known about non-target-site resistance in *Lolium* spp. to acetyl CoA carboxylase, acetohydroxyacid synthase, microtubule assembly, photosystem II, 5-enolpyruvylshikimate-3-phosphate synthase, glutamine synthetase, very-long chain fatty acids, and photosystem I inhibitors. We suggest research topics that need to be addressed, as well as strategies to further our knowledge and uncover the mechanisms of non-target-site resistance in *Lolium* spp.

Keywords: altered herbicide translocation, herbicide metabolism, multiple-herbicide resistance, cross-herbicide resistance, ryegrass, *Lolium rigidum* Gaud, *Lolium multiflorum* (Lam.), *Lolium perenne* (L.)

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***Correspondence:**
Caio A. C. G. Brunharo
caio.brunharo@oregonstate.edu

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INTRODUCTION

The *Lolium* genus contains many species of economic importance. *L. perenne* L. ssp. *perenne* (*L. perenne*), *L. perenne* L. spp. *multiflorum* (*L. multiflorum*), and *L. rigidum* are of particular relevance due to their widespread presence globally. These three species (hereinafter referred collectively to as *Lolium* spp.) are diploid ($2n = 2x = 14$), obligate outcrossing, and interfertile grass species that are widely planted for cover crop, turf, and pasture. These species are also considered weeds of agricultural and non-agricultural areas, and exhibit a distinctive ability to rapidly adapt to different environments.

Weed control is one of the most important components of cropping systems that results in significant yield and financial loss to growers if not properly performed. This scenario is exacerbated by the evolution of herbicide resistant weed populations, with 514 unique cases reported globally (Heap, 2020). Because of the overreliance on herbicides as the main weed management tool, resistance to multiple herbicide families within a single weed population is often documented (Neve et al., 2004). Multiple resistance represents a challenge to broad crop acreage production systems that depend on chemical weed management because of the lack of new herbicide molecules being marketed and the additional costs associated with non-chemical control methods.

Herbicide resistance mechanisms in weeds are typically classified in two categories: (a) modifications in the herbicide target enzyme (target-site resistance; TSR) and (b) mechanisms not involving the target enzyme (non-target-site resistance; NTSR). TSR is typically conferred by single major-effect alleles, whereas NTSR are believed to be conferred by multiple small-effect alleles (Jasieniuk et al., 1996; Délye, 2013), although this is not necessarily always the case (Yu et al., 2009b).

Physiological and biochemical alterations have been observed in weeds with NTSR, such as reduced herbicide absorption and translocation (Koger and Reddy, 2005), enhanced herbicide metabolism (Hall et al., 1997), and herbicide sequestration to the vacuole (Ge et al., 2010). However, the underlying physiological, biochemical, and genetic alterations conferring herbicide resistance is poorly understood.

Herbicide resistance in *Lolium* spp. populations has been widely documented. There are at least 125 reports of herbicide resistance in this genus to date, where multiple- and cross-resistance represent approximately 40% of the reports (Heap, 2020). In some regions of the world where environmental conditions for *Lolium* spp. development are ideal and there is an overreliance on herbicides as the main weed management tool, proportion of populations with multiple- and cross-resistance may be as high as 61% (Bobadilla, 2019). Herbicide resistance in *Lolium* spp. has been reported to 14 mechanisms of action, with an example of one population of *L. rigidum* from Australia with evolved resistance to seven mechanisms of action (HRAC/WSSA numbers 1, 2, 3, 8, 15, 13, and 23) (Burnet et al., 1994). *Lolium* spp. populations have evolved a variety of resistance mechanisms, including enhanced herbicide metabolism, reduced herbicide absorption and translocation, and protection-based resistance. Therefore, comprehensive reviews on the mechanisms of NTSR in *Lolium* spp. are needed.

In this article, we first provide an overview of NTSR mechanisms in weeds, with focus on grass species. We then review seminal and recent studies on NTSR in *Lolium* spp. It was not our goal to detail every single case of suggested NTSR in *Lolium* spp. Rather, we focused our efforts to compile the most relevant studies on NTSR in *Lolium* spp., what is known about the resistance mechanisms, and provide suggestions on how we can further our understanding of NTSR.

NTSR MECHANISMS IN WEEDS

Reduced Herbicide Absorption

Upon herbicide application, herbicide droplets must land on the leaf surfaces and overcome a number of barriers before cellular uptake. This passive process largely depends on leaf surface characteristics, herbicide chemical properties, and their interactions. Is this review, we distinguish herbicide absorption from cellular uptake, where the former is the process of overcoming the physical barrier of leaves (i.e., cuticle) before the herbicide reaches the apoplast, and the latter is the movement of herbicide from the apoplast into plant cells. Herbicide resistant populations may exhibit reduced herbicide absorption, which is characterized by a reduction in the penetration through the cuticle before reaching the epidermis (**Figure 1**), whereas cell walls do not pose a considerable resistance to cellular uptake (Sterling, 1994). Reduced absorption is not a common NTSR mechanism, but has been documented in both eudicots and monocots to the herbicide groups synthetic auxins and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors, resulting in low resistance levels (Kohler et al., 2004; De Carvalho et al., 2012).

Reduced Translocation and Vacuolar Sequestration

Most herbicides must translocate from their absorption site in order to control weeds. Therefore, alterations of translocation patterns can diminish herbicide efficacy (**Figure 2**). Herbicide resistance due to reduced translocation has been documented in grass weed species, such as *Lolium* spp. and *Chloris elata* (Wakelin et al., 2004; Yu et al., 2007, 2009a; Bostamam et al., 2012; González-Torralva et al., 2012; Brunharo et al., 2016). The underlying genetic and physiological basis of this NTSR mechanism remains poorly understood (Yuan et al., 2007; Ge et al., 2010, 2014).

Plant vacuoles are organelles that have central roles in the cell homeostasis, are involved in osmotic adjustment, are reservoirs for ions and metabolites, and storage of xenobiotics (Marty, 1999). Studies have shown that transporters such as ATP-binding cassettes (ABC) are possibly involved in herbicide movement into the vacuoles (Nol et al., 2012; Ge et al., 2014; Tani et al., 2015). Because many herbicides must reach a target site localized within specific organelles, the vacuolar sequestration may prevent the herbicides from reaching the target site, as well as symplastic movement of the herbicide molecules.

Reduced herbicide translocation as a NTSR mechanism varies with environmental conditions, in particular temperature. Studies have shown that low temperature regimes can reduce the resistance levels by affecting the kinetics of vacuole sequestration (Devine et al., 1983; Vila-Aiub et al., 2005; Shaner, 2009). A paraquat-resistant *L. multiflorum* biotype from California, for instance, exhibited a GR_{50} (herbicide dose required to reduce plant biomass by 50%) 21 times greater when grown at 30/24°C than at 16/10°C. This population also exhibited enhanced protection against reactive oxygen species (ROS) (Brunharo and Hanson, 2019).

Herbicide Metabolism

Herbicide metabolism refers to the degradation of herbicide molecules by endogenous plant enzymes. In some instances, this type of NTSR is non-specific, when a single enzyme may inactivate one or more herbicide within the same or different chemical classes (Iwakami et al., 2014b; Yu and Powles, 2014). Many aspects of the herbicide detoxification process are still unknown; however, key enzymes have been identified. Metabolism-based herbicide resistance occurs due to the increased activity of enzymes such as cytochrome P450's (Vila-Aiub et al., 2005; Yun et al., 2005; Busi et al., 2011; Iwakami et al., 2014a), glutathione S-transferases (GST's) (Reade et al., 2004; Cummins et al., 2011; Chronopoulou et al., 2017;

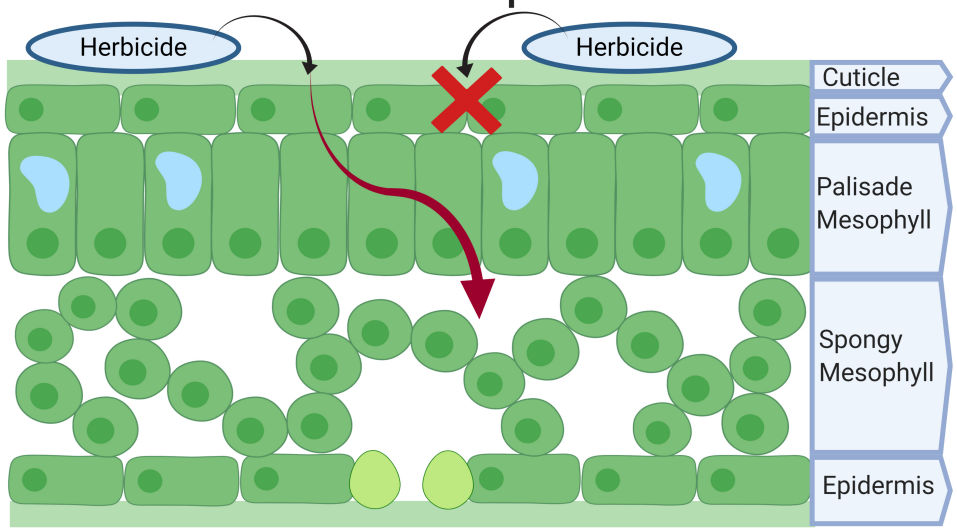

FIGURE 1 | Herbicide absorption in *Lolium* spp. Herbicide molecules must overcome the cuticle and epidermis to reach the apoplast before cellular uptake. X represents a halt in the herbicide absorption and the red arrow represents the pathway to the mesophyll that the herbicide molecules would normally have without a reduction in absorption.

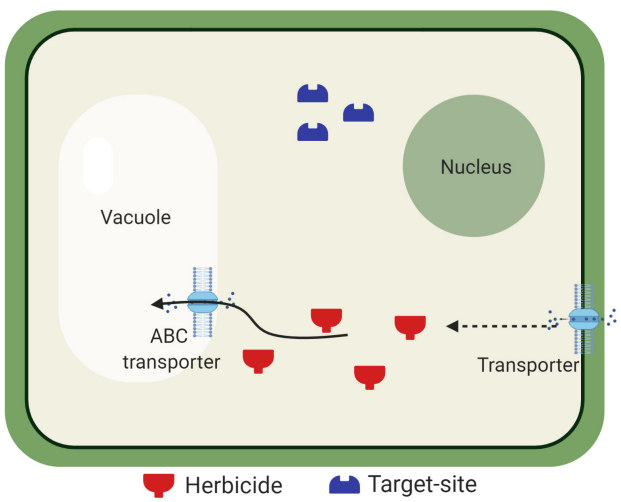

FIGURE 2 | Reduced herbicide translocation due to vacuolar sequestration. After herbicide cellular uptake, herbicide molecules must reach the target site. Tonoplast-bound transporters are believed to be involved in the vacuolar sequestration of herbicides in resistant *Lolium* spp. populations, preventing the herbicide from inhibiting its target enzyme. Transporters are also located in the plasma membrane for apoplast sequestration.

Dücker et al., 2019), ABC transporters (Rea et al., 1998; Yuan et al., 2007; Tani et al., 2015), and glucosyltransferases (GT) (Cotterman and Saari, 1992; Yuan et al., 2007; Powles and Yu, 2010). The genetic mechanisms of the altered enzyme activity is not fully understood. Several hypothesis, however, may be inferred: (i) genetic modifications within the genes that encode metabolizing enzymes are involved, enhancing their activity; (ii) genetic modifications outside of the genes (e.g., in the promoter region or intragenic regions) enhance gene expression and, consequently, number of enzymes available to degrade herbicides; (iii) epigenetic changes occurred due to previous stressors (e.g., low rates of herbicides) that altered the epigenome, enhancing the expression of genes that encode metabolizing enzymes; (iv) and post-translational modifications of proteins enhance enzyme activity.

Herbicide metabolism can be divided into three phases (**Figure 3**). The process starts after herbicide cellular uptake. Hydrophobic herbicide molecules are oxidized to a more hydrophilic metabolite, generally by P450's (e.g., hydrolysis, oxidation, etc.; Phase I). Once the herbicide molecule is more hydrophilic, a conjugation reaction of the herbicide molecule may take place, and the herbicidal activity and hydrophobicity are further reduced (Phase II). Herbicides that already possess hydrophilic properties may be directly subjected to Phase II. Lastly, transport enzymes may recognize conjugated herbicide molecules before storage into vacuoles and cell walls (Phase III) (Yuan et al., 2007; Délye, 2013; Yu and Powles, 2014; Jugulam and Shyam, 2019). Some researchers also recognize a Phase IV of the herbicide metabolism process, where stored molecules are later utilized for plant metabolism (Rosinger et al., 2012).

Cytochrome P450's are oxidoreductase enzymes that catalyze the hydroxylation, oxidation, and reduction, among other reactions, of substances in many organisms and are known to play a significant role in protecting plants from abiotic and biotic stresses (Mizutani and Sato, 2011). Plants have over 40 different families of cytochrome P450's and are divided into four categories according to their primary function. P450 gene sequences occupy approximately 1% of the plant genome, reflecting their importance in plant defense and other functions

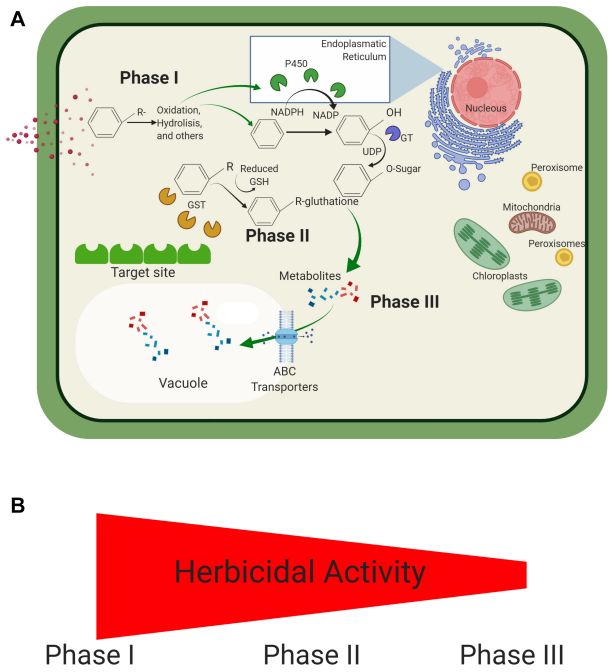

FIGURE 3 | Herbicide metabolism in *Lolium* spp. An herbicide may be metabolized in three distinct processes, which typically occur in consecutive order. **(A)** Initially, the herbicide is subjected to a redox reaction to increase its hydrophilicity (Phase I). This metabolized herbicide may now be subjected to further processing in Phase II (e.g., conjugation). Metabolism may be concluded with the storage of metabolized compounds (Phase III). **(B)** The herbicidal activity decreases with the consecutive processing of herbicides.

such as synthesis and catabolism of plant hormones (Nelson and Werck-Reichhart, 2011; Mizutani, 2012; Pandian et al., 2020).

According to Guengerich (2018) and Pandian et al. (2020), P450 herbicide detoxification is known to happen in five steps: the first step consists in the herbicide binding to the heme group. In the second step, the substrate binding induces the electron transfer from NADPH by P450 reductase; the third step consists in oxygen binding to the ferrous cytochrome forming a complex; in the fourth step, the P450 reductase will release another set of electrons to the ferrous cytochrome-dioxygen complex forming a short lived "peroxo" complex that is rapidly protonated forming a water and an iron–oxo complex; the last step consists on the complex binding to the organic herbicide molecules forming an oxidized product.

Many studies that reported enhanced herbicide metabolism as the resistance mechanism did so by indirect means. Typically, a P450 inhibitor is applied either before or with the herbicide being tested, with the expectation that the P450 inhibitors would reverse the resistance phenotype. For instance, Christopher et al. (1994) pre-treated chlorsulfuron-resistant *L. rigidum* with malathion (a P450 inhibitor) and observed that the resistant population responded similarly to the susceptible. More recently, cytochrome P450s were identified to confer NTSR resistance in *Lolium* spp. populations from Argentina after the authors pre-treated plants with malathion, 1-aminobenzotriazole, and piperonyl butoxide. P450 inhibitors are a widely used approach to identify the role of P450s in herbicide resistance (Busi et al., 2017; Zhang et al., 2017; Yanniccari et al., 2020).

The enzyme super-family of GSTs is also involved in herbicide detoxification in plants. In maize, for instance, GST's represent more than 1% of soluble proteins in leaves (Edwards et al., 2000). GST's catalyze the conjugation of many hydrophobic and electrophilic substrates with the tripeptide glutathione (Edwards et al., 2000). GST's are likely to be involved in the compartmentalization of herbicides by conjugating glutathione with herbicide molecules and facilitating the recognition of glutathione transporters making them potential participants in reduced translocation-based resistance (Reade et al., 2004).

The ABC superfamily is another large group of proteins that is responsible to mediate a wide range of transport functions in plants (Theodoulou, 2000). ABC transporters can play a role in the transport and movement of many compounds such as peptides, sugars, lipids, heavy metal chelates, polysaccharides, alkaloids, steroids, inorganic acids, and glutathione conjugates; these transporters can be highly specific and able to transport a large variety of compounds (Higgins, 1992). Research has shown that ABC transporters may actively transport and compartmentalize herbicide conjugates and metabolites (Powles and Yu, 2010; Gaines et al., 2020). ABC transporters have been hypothesized to be involved in the glyphosate resistance mechanism in *Lolium* spp. (Ge et al., 2012).

Glycosylation mediated by GT's may alleviate stresses caused by xenobiotics in plants (Bowles et al., 2005). In grasses, GT's are known to be responsible for their tolerance to synthetic auxins via glycosylation (Devine et al., 1993). In many *Lolium* spp. studies, genes that encode GT's have been identified as potential players in the resistance mechanisms to several herbicides (Gaines et al., 2014; Busi et al., 2018; Dücker et al., 2019).

Protection-Based Resistance

Protection-based herbicide resistance is conferred by endogenous enzymes that counteract the damaging effect of reactive molecules that were elicited by the action of an herbicide. The most widely studied enzymes are those of the Halliwell-Asada cycle, which are involved in the protection of plant cells against oxidative damage (Délye, 2013), and include superoxide dismutase, ascorbate peroxidase, and glutathione reductase. Many herbicides elicit the overproduction of ROS which can induce oxidation of proteins, DNA, and lipids, resulting in cellular damage and causing cellular leakage. A plant that can avoid or reduce the presence of ROS can minimize the stress caused by herbicides. An *Alopecurus myosuroides* population with multiple resistance to photosystem II (PSII) and acetyl CoA carboxylase (ACCase) inhibitors exhibited an enhanced activity of enzymes involved in the cellular protection against toxic organic hydroperoxides (Cummins et al., 1999). However, there are few documented cases of protection-based resistance, and detailed information on its role as a secondary mechanisms of resistance is limited. If individuals in a population exhibit enhanced protection against ROS, then it would be expected that reduction in efficacy of many herbicide classes would be observed.

HERBICIDE RESISTANCE IN *Lolium* spp. AND THEIR MECHANISMS OF NTSR

Resistance to ACCase Inhibitors

Herbicides in the aryloxyphenoxy-propionate (FOP's), cyclohexanedione (DIM's), and phenylpyrazoline (DEN) chemical families (HRAC/WSSA Group 1) inhibit ACCase, an enzyme in the biosynthetic pathway that produces fatty acids, which are required for lipid production needed for cell membranes (Hoppe, 1989). The binding site is a 400-amino acid fragment of the carboxyltransferase (CT) domain in ACCase (Nikolskaya et al., 1999; Takano et al., 2021). Herbicides in these families are extremely effective for grass control and in general, the chloroplastic ACCase from broadleaf plants is not sensitive to ACCase inhibiting herbicides (Konishi and Sasaki, 1994). In tolerant grasses, the herbicides are metabolized to non-toxic products or have insensitive ACCase (Shimabukuro, 1985; Duke and Kenyon, 1988; Zimmerlin and Durst, 1992). Some of the herbicides are selective and can be used in cereal crops while others are non-selective. For example, wheat (*Triticum aestivum*) is tolerant to diclofop-methyl and clodinafop-propargyl but not to fluazifop-p-butyl, quizalofop-p-ethyl, clethodim, and sethoxydim (Shaner, 2014). In susceptible plants and in wheat, diclofop-methyl is bioactivated by hydrolysis to form the phytotoxic diclofop acid (**Figure 4**). In wheat, the acid is detoxified by aryl hydroxylation catalyzed by a P450 monooxygenase followed by glucosylation to produce a non-toxic glucose conjugate (Shimabukuro, 1985).

Resistance to ACCase inhibitors in *Lolium* spp. is common with reports from all continents except Antarctica. Diclofop resistant *L. rigidum* was reported in Australia in 1982 (Heap and Knight, 1982) and *L. multiflorum* in Oregon in 1987 (Stanger and Appleby, 1989). Subsequently, resistance has been reported in *Lolium* spp. to all herbicides in Group 1. Although, resistance to clethodim is less common. Resistance to one of the herbicides in this group does not necessarily lead to cross-resistance with other members of the group. Target site resistance is due to a single point mutation in the ACCase gene with at least five different mutations reported with some mutations providing resistance to all three families (Powles and Yu, 2010; Takano et al., 2021).

Non-target-site resistance to diclofop in *L. rigidum* was reported in Australia in 1991 (Holtum et al., 1991). The researchers did not believe that the 10% difference in metabolism between resistant and susceptible plants was enough to produce a 30-fold difference in sensitivity at the whole plant level. The authors suggested that metabolism plus membrane repolarization might be responsible for resistance. Other researchers also proposed that membrane depolarization results from the application of ACCase inhibitors and that resistant plants were able to recover from this effect (Devine and Shimabukuro, 1994; Shimabukuro and Hoffer, 1997). However, the membrane depolarization observed in plants treated with ACCase inhibitors may be considered a secondary effect, as was determined the target is the CT-domain of ACCase (Nikolskaya et al., 1999). Further research on resistant *Lolium* spp. populations showed that enhanced metabolism via P450 followed by conjugation by GST enzymes were responsible for resistance (Preston et al., 1996; Preston and Powles, 1998; Cocker et al., 2001; De Prado et al., 2005). De Prado et al. (2005) also reported reduced absorption of diclofop and greater epicuticular wax density in one resistant biotype of *L. rigidum*.

Resistance to AHAS Inhibitors

There are five herbicide families (HRAC/WSSA Group 2) that inhibit acetohydroxyacid synthase (AHAS), also referred to as acetolactate synthase (ALS), the first enzyme in the biosynthetic pathway for the production of the branched chain amino acids, isoleucine, leucine, and valine. The families are imidazolinones, pyrimidinyl-thiobenzoates, sulfonylamino-carbonyl-triazolinone, sulfonylureas, and triazolo-pyrimidines.

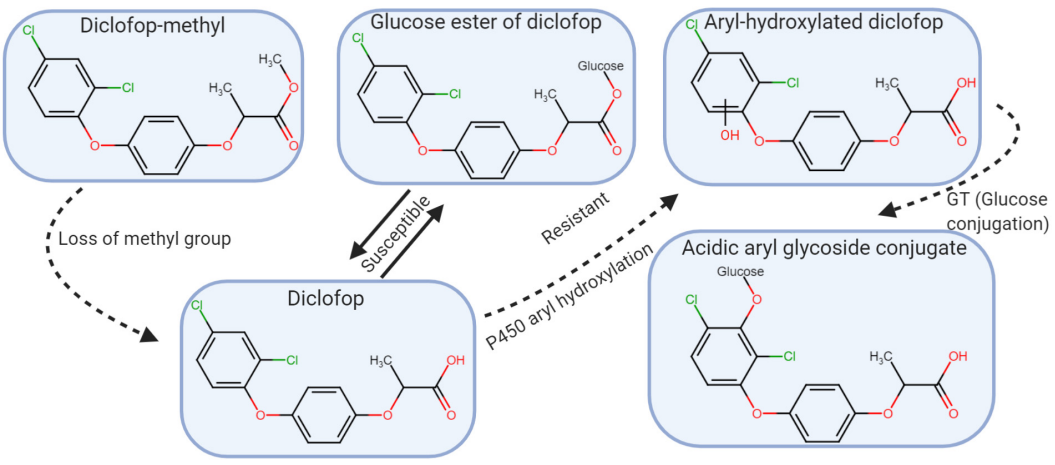

FIGURE 4 | Diclofop-methyl metabolism in susceptible and resistant plants. Diclofop-methyl is demethylated, and converted to the active form of the herbicide. In resistant plants, diclofop undergoes an aryl hydroxylation reaction likely mediated by P450, followed by a conjugation reaction to acidic aryl glycoside of diclofop. In susceptible plants, diclofop is reversibly converted to a glucose ester conjugate (modified from Powles and Holtum, 1994).

The herbicides are used in nearly all cropping systems with major differences in their selectivity, spectrum of control, and residual activity.

Similar to the ACCase inhibitor herbicides, resistance to AHAS inhibitors in *Lolium* spp. has been reported on every continent except Antarctica. Most of the resistant populations were identified in cereal cropping systems with some identified in other crops or in non-crop areas such as roadsides. Initially, TSR was reported to be the most common resistance mechanism with multiple different point mutations responsible for resistance (Tranel and Wright, 2002). However, there are many cases of NTSR AHAS resistance in *Lolium* spp. reported to be due to enhanced metabolism. Further, TSR or NTSR to one AHAS inhibiting herbicide does not necessarily endow resistance to another herbicide even within the same chemical family.

In studies conducted on *L. rigidum*, metabolism of chlorsulfuron, a sulfonylurea herbicide, occurred more quickly in the resistant biotype compared to the susceptible biotype (Christopher et al., 1991, 1992). Using high-pressure liquid chromatography (HPLC), the major metabolite co-eluted was the glucose-conjugate metabolite previously identified in chlorsulfuron tolerant wheat (**Figure 5**; Christopher et al., 1991). In another study using a different chlorsulfuron resistant *L. rigidum* biotype, the major metabolite identified was the glucose conjugate of hydroxyl-chlorsulfuron (Cotterman and Saari, 1992). In the resistant biotype, 50% of the chlorsulfuron was metabolized within 2 h compared to 10% in the susceptible biotype. The percentage of the glucose conjugate occurred more rapidly and to a greater level in the resistant biotype compared to the susceptible biotype. The researchers further showed that chlorsulfuron metabolites were not AHAS inhibitors so the differences in rate and level of chlorsulfuron metabolism were responsible for resistance. In many other studies, resistance due to enhanced metabolism resistance was based on indirect evidence. In these studies, a cytochrome P450 inhibitor, such as malathion (Preston et al., 1996; Yu et al., 2009a) or chlorpyrifos (Liu M. et al., 2016), was applied. In these studies, resistance was overcame with the addition of the P450 inhibitor, implicating herbicide metabolism as the mechanism of resistance.

Resistance to Microtubule Assembly Inhibitors

Herbicides that inhibit the assembly of microtubules do so by binding to α or β-tubulin (HRAC/WSSA Group 3) leading to loss of microtubule structure and function in the process of mitosis. Microtubules are required for the spindle apparatus, which separates sister chromatids during mitosis (Molin and Khan, 1997). This loss of function prevents cell division and cell wall formation. The most widely used herbicides with this mechanism of action are in the dinitroaniline chemical family, which includes the herbicide trifluralin.

Trifluralin resistant *L. rigidum* populations have been reported in Australia. In most cases, the resistant populations were found in cereal cropping systems. Several of the populations were reported to be resistant to other herbicides. In some cases, the mechanism of resistance was due to single point mutation in the α-tubulin gene, where four unique point mutations have been identified that provide resistance to trifluralin (Chen et al., 2018; Chu et al., 2018; Fleet et al., 2018).

The only reports of trifluralin NTSR are from studies conducted in populations collected in Western Australia, which confirmed NTSR via enhanced metabolism (Chen et al., 2018). Using thin-layer chromatography (TLC) and HPLC analyses, more trifluralin was metabolized in a resistant population compared to a susceptible population. Because metabolites were not identified, the specific degradation pathway was not determined. However, in previous studies with one of the resistant populations, resistance was reversed when a P450 inhibitor was applied indicating that a P450 enzyme might be involved with the enhanced metabolism (Busi et al., 2017). In addition, in this study, some plants evolved both TSR and NTSR to trifluralin.

Resistance to Photosystem II Inhibitors

Several different herbicide chemical classes including triazines, triazinones, and ureas (HRAC/WSSA Groups 5 and 6) inhibit Photosystem II (PSII). The PSII complex is located within the thylakoid membranes of chloroplasts and contains two proteins, D2 and D1 (Fuerst and Norman, 1991). Once a PSII inhibiting herbicide binds, it blocks the transfer of electrons from plastoquinone Q_A in D2 to plastoquinone Q_B in D1, which prevents CO_2 fixation and production of ATP and NADPH. Blocking electron transport leads to production of ROS, which destroy cell integrity.

The first documented case of herbicide resistance was in the dicotyledonous species common groundsel (*Senecio vulgaris* L.) to the PSII inhibitor simazine, a triazine herbicide (Ryan, 1970). Most often, the mechanism of resistance to PSII inhibitors is reported to be a mutation in the D1 protein in the PSII complex. However, there are some cases where resistance is NTS. In these cases, enhanced metabolism is reported to be responsible for resistance. Photosystem II inhibitor resistant *L. rigidum* populations have been reported in crop and non-crop sites in Australia, Israel, and Spain and *L. multiflorum* populations in United Kingdom cereal crops (Heap, 2020).

Metribuzin (HRAC/WSSA Group 5) is in the triazinone chemical family of PSII inhibitors. Metribuzin controls both grass and broadleaf weeds. In some tolerant species such as wheat, metribuzin is detoxified to polar N-glucoside metabolites (Devlin et al., 1987). Metribuzin resistance in a multiple-resistant *L. rigidum* population in Australia was due to enhanced metabolism (Ma et al., 2020). In a time course study, unidentified polar metabolites of metribuzin were found in both susceptible and resistant plants at each time point but were greater in the resistant plans (Busi et al., 2017). Based on the results of a dose response study with the addition of a P450 inhibitor, which reversed resistance, the authors suggested that the mechanism of resistance likely involved cytochrome P450 monooxygenases.

Chlorotoluron (HRAC/WSSA Group 5) is in the substituted urea chemical family of PSII inhibiting herbicides but has a different binding behavior compared to other herbicide classes in Group 5 (Shaner, 2014). Chlorotoluron is used to control

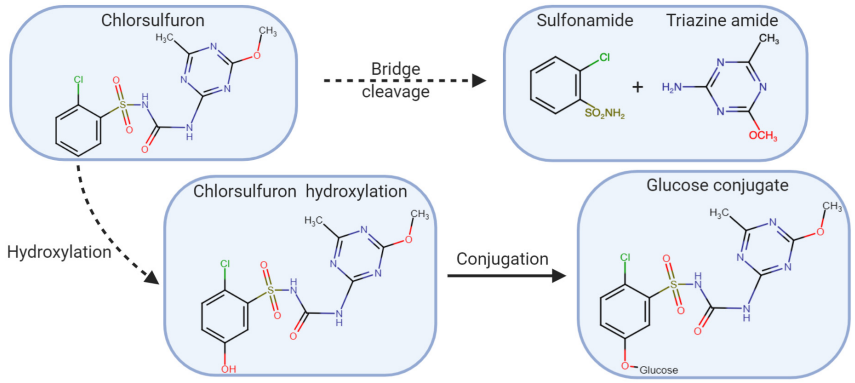

FIGURE 5 | Chlorsulfuron metabolism in *Lolium rigidum*. The herbicide may be hydroxylated followed by conjugation with glucose, or cleaved, producing sulfonamide and triazine amide (adapted Cotterman and Saari, 1992).

grass and broadleaf weeds in cereals. In tolerant plants, the major degradation is through N-dealkylation and oxidation of the ring-methyl group with conjugation to glucose (Gonneau et al., 1988).

Similar studies on the mechanism of chlorotoluron resistance in *L. rigidum* populations were conducted in Australia (Burnet et al., 1993; Preston and Powles, 1996) and Spain (De Prado et al., 1997). Based on HPLC analysis, Burnet et al. (1993) found that resistant plants metabolized chlorotoluron more quickly than the susceptible plants. De Prado et al. (1997) used TLC and reported greater metabolism at 48 hr in the resistant versus susceptible plants. Both groups conducted studies using monooxygenase inhibitors to overcome resistance, which supported the premise that cytochrome P450 enzymes could be involved in degradation. In a follow up study on one of the resistant Australian populations, chlorotoluron was metabolized via two paths, which resulted in either ring-methyl hydroxylation or N-demethylation (Preston and Powles, 1997). In light, metabolism via ring-methyl hydroxylation increased significantly while N-demethylation did not. The products of ring-methyl hydroxylation were conjugated to glucose. The results indicated that the ring-hydroxylation was the major detoxification pathway and that N-demethylation was less important. The metabolism via both pathways was greater in chlorotoluron resistant plants than in susceptible plants. The authors suggest that two different enzymes are involved with enhanced metabolism of the resistant biotype because of the differences in the induction of the two pathways in response to light.

Resistance to Glyphosate

Glyphosate [*N*-(phosphonomethyl)glycine, HRAC/WSSA Group 9] is the most widely used herbicide in the world in agricultural and non-agricultural areas. It inhibits EPSPS, preventing biosynthesis of aromatic amino acids for plant metabolism (Shaner, 2014). Glyphosate uptake by plant cells may be active or passive, with the active uptake being facilitated by membrane-bound phosphate transporters (Hetherington et al., 1998). Several properties make glyphosate a unique and important tool: it is a non-selective, systemic, slow-acting, post-emergence, and relatively non-expensive herbicide (Duke et al., 2018). Glyphosate is hydrophilic (log K_{ow} at pH 7 = of −3.1), a weak acid, and exhibits slow metabolic degradation in most plants or not at all, which makes it possible for glyphosate to be transported through the phloem and the xylem and move to meristems where amino acid synthesis is most required (Duke et al., 2018). In 1996, *L. rigidum* was the first species to have a confirmed glyphosate resistant population (Pratley et al., 1996).

Most cases of NTSR to glyphosate in *Lolium* spp. are due to reduced translocation, with more than 20 reports to date in several countries, including Australia, Brazil, Chile, France, Italy, Japan, New Zealand, Portugal, Spain, and the United States (Mississippi and Oregon) (Ferreira et al., 2006; Michitte et al., 2007; Perez-Jones et al., 2007; Yu et al., 2007; Nandula et al., 2008; Ge et al., 2012; Ghanizadeh et al., 2015b; Fernández-Moreno et al., 2017; Kurata et al., 2018). Some authors also reported lower spray retention and foliar uptake from the abaxial leaf surface, along with reduced translocation (Michitte et al., 2007).

Reduced glyphosate translocation in resistant plants keeps the herbicide in the source leaves, away from the meristematic tissue, enabling survival after treatment (Kurata et al., 2018). Susceptible biotypes commonly translocate glyphosate out of the treated leaves into non-treated leaves, meristematic tissues, stems and roots (Lorraine-Colwill et al., 2002; Wakelin et al., 2004; Perez-Jones et al., 2007; Yu et al., 2009a). Different populations exhibit a wide range of resistance levels, ranging from three- to 25-fold compared to susceptible populations (Ghanizadeh et al., 2015b; Kurata et al., 2018).

Reduced glyphosate movement in glyphosate resistant plants may occur *via* four mechanisms: (i) modification in a putative phosphate transporter located in the plasma membrane, (ii) an active transporter pumps glyphosate into the vacuole, (iii) glyphosate pumped out of the cell into the apoplast through an active transporter, (iv) glyphosate pumped out of the chloroplast by a transporter in the chloroplast envelope (Shaner, 2009). However, to date, these mechanisms remain hypothetical, and no transporter has been identified to confer glyphosate resistance in weeds.

A modification in a phosphate carrier protein has been proposed as a resistance mechanism to glyphosate (Shaner, 2009; Roso and Vidal, 2010). It has been shown that glyphosate does not readily move across a laboratory made semi-permeable membrane (Takano et al., 2019) and cellular uptake may be inhibited in the presence of phosphate (Hetherington et al., 1998). These results provide evidence that glyphosate is taken up by the cell through a phosphate transporter. Therefore, a putative modification in such a transporter would keep glyphosate out of the cell. However, a possible modification in the carrier has not been found to date in *Lolium* spp.

The second possible mechanism, a transporter pumping glyphosate into the vacuole has been the hypothesis with the most evidence found to date. In a study using ^{31}P nuclear magnetic resonance, vacuolar sequestration of glyphosate in populations of *Lolium* spp. from four different countries was strongly correlated with reduced translocation, and thus, reduced entry of glyphosate into the phloem (Ge et al., 2012). The authors concluded that glyphosate sequestration into the vacuole appeared to be unidirectional, meaning that once inside the vacuole, efflux through the tonoplast does not seem to be significant. The authors hypothesized that glyphosate is transported into the vacuole through an unidentified tonoplast-bound ABC transporter (Ge et al., 2012; Sammons and Gaines, 2014). To date, only a few studies have investigated the vacuolar sequestration and its association with reduced translocation of glyphosate. However, a few candidate genes have been identified. Glyphosate movement across the tonoplast is reduced under low temperatures (Ge et al., 2011). Studies in *Lolium* spp. have used low temperature treatments after glyphosate application as indirect evidence that glyphosate was sequestered into the vacuole (Vila-Aiub et al., 2013; Ghanizadeh et al., 2015a). *Lolium* spp. populations evaluated in other studies had reduced herbicide translocation as the mechanism of resistance when grown at ambient temperatures (Lorraine-Colwill et al., 2002) of 26/12°C (Ghanizadeh et al., 2015b). When grown at 9°C after glyphosate application, the resistant population responses were similar to the susceptible population. In comparison, a glyphosate resistant *L. multiflorum* with an *EPSPS* $Pro_{106}Ser$ amino acid substitution was not made sensitive to glyphosate with cold acclimation (Collavo and Sattin, 2012; Sammons and Gaines, 2014). However, since low temperature is also the same method used to identify possible metabolism based resistance, more research would need to be done to rule out this hypothesis and elucidate the effects of temperature on the vacuolar sequestration of glyphosate.

Although most studies of resistant populations with reduced translocation did not further investigate the underlying genetic basis of the NTSR, it is very likely that they also had vacuolar sequestration, as enhanced glyphosate metabolism has rarely been identified to date (however, see Pan et al., 2019; McElroy and Hall, 2020).

Reduced glyphosate translocation generally results in higher resistance levels than alterations in the EPSPS enzyme (Preston and Wakelin, 2008; Bostamam et al., 2012). It has been suggested that two or more mechanisms of resistance in the same population, can result in a higher level of resistance (Ghanizadeh et al., 2015b). As *Lolium* spp. are obligate outcrossing species, different mechanisms of resistance and resistance to different herbicides may accumulate due to cross-pollination (Yu et al., 2007).

No evidence of glyphosate being pumped out of the cell into the apoplast, nor being pumped out of the chloroplast envelope has been found to date. Glyphosate transport through membranes has been observed as being unidirectional by importers (Ge et al., 2013). Once glyphosate enters the chloroplast, it has been assumed that it cannot return to the cytoplasm (Sammons and Gaines, 2014). An upregulated gene was found to be related to ABC transporter A family member 7 (*ABCA7*) in a NTSR glyphosate resistant *L. multiflorum* population (Cechin et al., 2020), which its subcellular location is in the plasma membrane in *Arabidopsis thaliana* (Benschop et al., 2007). Further validation studies could help determine if the identified transporter gene is responsible for glyphosate resistance.

Enhanced glyphosate metabolism has not been found to be a resistance mechanism in *Lolium* spp.; however, Fernández-Moreno et al. (2017) found that susceptible and resistant populations of *L. perenne* and *L. multiflorum* metabolized glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate. The authors concluded that the final concentrations of the metabolites were small and unlikely to be biologically meaningful. AMPA is a very weakly phytotoxic compound (Gaines et al., 2020) and glyoxylate is a non-toxic compound (Rueppel et al., 1977), therefore rapid degradation to those substances should provide glyphosate resistance. In a RNA-seq study comparing a susceptible and a NTSR population, the candidate gene list included genes related to glycosyltransferases (Cechin et al., 2020). Glycosyltransferases are important for crop tolerance; however, their role in herbicide resistance in weeds is still not well understood and glucosylation of glyphosate as a mechanism of NTSR has yet to be identified (Rigon et al., 2020). Future studies with reverse genetics to evaluate candidate genes are required.

Resistance to Glufosinate

Glufosinate (HRAC/WSSA Group 10), the only member of this herbicide group, controls weeds by inhibiting the glutamine synthetases, key enzymes in the nitrogen assimilation in plants. Inhibition of glutamine synthetase reduces the amount of amino donors for the glycolate pathway, breaking the transamination reaction of glyoxylate to glycine in the photorespiratory cycle (Wild and Wendler, 1993). This imbalance leads to accumulation of glyoxylate, which is a strong inhibitor of the ribulose-1,5-bisphosphate carboxylase activase, necessary for the proper functioning of ribulose-1,5 bisphosphate carboxylase/oxygenase. Consequently, photosynthesis is inhibited (Wendler et al., 1992; Wild and Wendler, 1993; González-Moro et al., 1997), causing accumulation of ROS and cell death (reviewed by Hess, 2000, and more recently by Takano and Dayan, 2020).

There are, overall, a limited number of glufosinate resistant weed populations, likely associated with the limited use of this herbicide until recent years. More recently, however, particularly because of patent expirations and increased adoption of glufosinate resistant crops, the number of resistant populations

has increased and this trend is likely to continue. Glufosinate resistance in *L. multiflorum* was first identified in 2009 in hazelnut (*Corylus avellana*) orchards in Oregon, where resistant populations exhibited up to 2.7-fold reduced response to glufosinate compared to a known susceptible population (Avila-Garcia and Mallory-Smith, 2011; Avila-Garcia et al., 2012). Later, research by Brunharo et al. (2019) indicated that there are multiple mechanisms of glufosinate resistance in the Oregon populations. The authors studied two resistant populations, one of them exhibited enhanced glufosinate metabolism, and the other did not. No differences in absorption, translocation of glufosinate, or differential gene expression of three GS isoforms studied were observed. The metabolites produced by glufosinate resistant *L. multiflorum* were not identified. Several plant species have been identified that may metabolize glufosinate, including tobacco and carrot (Dröge et al., 1992), producing several stable and unstable compounds with reduced herbicidal activity (Droge-Laser et al., 1994). Current research is underway to identify the genetic basis of glufosinate resistance in *L. multiflorum*.

Resistance to Very-Long Chain Fatty Acid Inhibitors

Very-long chain fatty acid (HRAC/WSSA Group 15) inhibitors (e.g., flufenacet, metolachlor, and pyroxasulfone) prevent biosynthesis of very-long chain fatty acid although a specific target enzyme or enzymes within the pathway have not been identified. Trenkamp et al. (2004) reported that flufenacet inhibits multiple elongases in the pathway.

Rapid metabolism of flufenacet via glutathione conjugation is found in tolerant crops with flufenacet-glutathione being the first major metabolite (Bieseler et al., 1997). Activity rates of GST were greater in maize, a tolerant crop, than in sensitive species, supporting the role of this enzyme in the breakdown of flufenacet in plants (Kreuz et al., 1989).

Resistance to flufenacet has been reported in *L. multiflorum* in France and United States (Gersdorf, 2009; Rauch et al., 2010; Liu M. et al., 2016; Bobadilla, 2019; Dücker et al., 2019). Most of the resistant populations were found in either cereal or grass seed cropping systems and were resistant to other herbicides (i.e., exhibited cross- and multiple-resistance). Liu M. et al. (2016) suggested that resistance in populations from Oregon was based on enhanced metabolism.

Pyroxasulfone resistance has been artificially created in *L. rigidum* populations under laboratory conditions after recurrent low-rate herbicide (Busi et al., 2012). These populations were subjected to three cycles of an increasing rate of pyroxasulfone, and the resistance phenotype has been attributed to an enhanced rate of herbicide metabolism (Busi et al., 2018). A field population of *L. rigidum* evolved pyroxasulfone resistance in Australia (Brunton et al., 2019).

Studies conducted by Dücker et al. (2019) found that flufenacet resistance in *L. multiflorum* populations from France, the United Kingdom, and Washington State, United States, was due to enhanced metabolism. Flufenacet was degraded more quickly in resistant plants than in susceptible plants with some variation among the susceptible and resistant tested populations (**Figure 6**). In sensitive populations at 22°C, times for 50% degradation (D_{50}) of flufenacet were 7 to 12 h whereas in the resistant populations the D_{50s} were 0.09 to 0.41 h. At 12°C, the D_{50s} were 18.5 to 46 h for the susceptible populations and 1.3 h for the resistant populations. A flufenacet-glutathione conjugate was found to be the first metabolite in the degradation pathway. GST activity was greater in the resistant plants than in susceptible populations. Two additional metabolites were identified in the resistant plants during the time course study. At 24 h, metabolites that were likely the result of secondary conjugation with malonyl or glycosyl were detected.

Resistance to Photosystem I Electron Diverters

Paraquat and diquat are non-selective herbicides (WSSA/HRAC Group 22) that function as preferential electron acceptors in the Photosystem I (PSI), where electrons from ferredoxin are diverted from their regular path, producing ROS that cause lipid peroxidation and tissue necrosis (Summers, 1980). Throughout this section, the focus will be given on paraquat, as more in-depth studies on the NTSR mechanisms for this herbicide are available.

Paraquat cellular uptake is facilitated by plasma membrane-bound polyamine transporters (Hart et al., 1992), likely because of the similar chemical structure the herbicide shares with these natural substrates (Fujita and Shinozaki, 2014). Once the plasma membrane barrier is overcome, paraquat must reach its target site located in the chloroplast, more specifically in the thylakoid membrane. It is unclear whether paraquat transport through the chloroplast's double-membrane, particularly the inner, less-permeable membrane, is passive or active. Results from Li et al. (2013) suggest that an L-type amino acid (LAT) transporter localized to the Golgi apparatus facilitates paraquat movement into the chloroplast. LAT transporters are involved in the intracellular movement of LAT, polyamines, and organocations in mammals (Jack et al., 2000), and the authors suggested that LAT transporters facilitate the movement of paraquat to the chloroplast.

Because paraquat does not have a target site enzyme associated with its mechanism of action, resistance to paraquat has always been associated with NTS. Resistance to paraquat has been proposed to be either because of vacuolar sequestration of the herbicide or enhanced protection against ROS, where the former typically confers higher resistance levels. Although there are many reports of differential response to PSI inhibitors in populations of *Lolium* spp. (Faulkner, 1974; Harvey et al., 1978), the first field-selected case of PSI resistance was not identified until 2002 (Yu et al., 2004).

Lolium rigidum was the first member of the *Lolium* spp. complex to exhibit PSI inhibitor resistance (Yu et al., 2004) from a vineyard in South Africa. The resistant population exhibited 30-fold reduced translocation compared to a known susceptible population. The authors suggested that the mechanism of paraquat resistance involved enhanced vacuolar sequestration of the herbicide, supported by the fact that resistance could be reversed by plant incubation under low temperatures, as is observed for paraquat resistance in other species (Purba et al., 1995). Later inheritance studies in other populations suggested

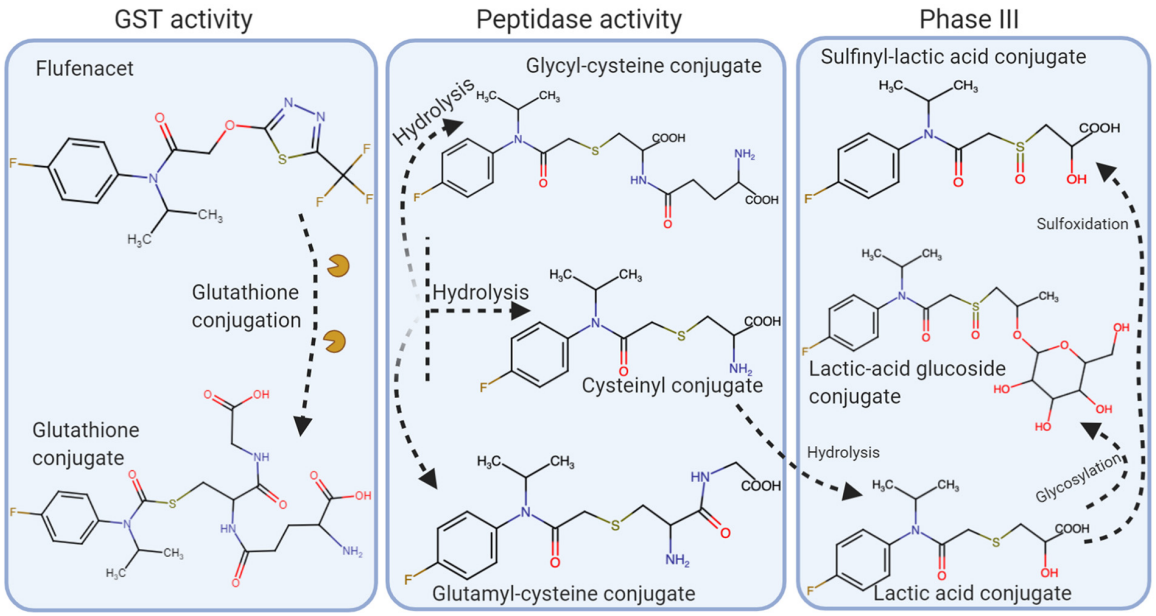

FIGURE 6 | Flufenacet metabolism in *Lolium* spp. Herbicide conjugation is initially performed by GST's. The conjugate molecule is hydrolyzed and processed by peptidases, which will eventually be further processed in the Phase III of the metabolism pathways (adapted from Dücker et al., 2019).

that a major nuclear gene confers paraquat resistance, as the phenotype followed Mendelian segregation (Yu et al., 2009b).

Paraquat resistance in *L. multiflorum* was first observed in 2015, in a population from a prune orchard in California (Brunharo and Hanson, 2017). No differences were observed in paraquat metabolism or absorption when the resistant and susceptible biotypes were compared when grown at 30/24°C (Brunharo and Hanson, 2019). However, significant differences in paraquat translocation were detected, where the resistant biotype translocated less paraquat than the susceptible in a light-manipulated environment. After paraquat application, the resistant biotype exhibited a transient inhibition of photosynthesis, suggesting a mechanism of response to the herbicide once inside the plant cells. Furthermore, at low paraquat doses, there was no damage observed to thylakoid membranes of treated plants, suggesting a constitutive mechanism to cope with the herbicide, whereas at higher paraquat doses significant damage was observed. The authors concluded that paraquat resistance was due to vacuolar sequestration of the herbicide, because pre-treatment of leaf tissues with a tonoplast-bound polyamine transport inhibitors reversed the resistance. When this population was acclimated to low temperatures 16/10°C, paraquat resistance was no longer observed (Brunharo and Hanson, 2019). This population also exhibited an enhanced ability to detoxify ROS. To the best of our knowledge, there are no reports of PSI resistance in *L. perenne*. The physiological mechanisms involved in the paraquat resistance reversal under low temperatures have not been elucidated. However, one could hypothesize that, if the resistance mechanism depends on enzyme kinetics of transport proteins, then low temperatures will reduce the rate of enzyme reactions.

STRATEGIES TO UNCOVER NTSR

Scientists have acquired a plethora of information on target-site resistance. The large amount of information on TSR may be attributed to the fact that, when herbicide resistance is believed to have evolved in a weed population, looking for changes in the target site can be successfully achieved relatively quickly in the laboratory today. Basic understanding of NTSR mechanisms, conversely, is still in its early stages of discovery, and limited advances on the genetic basis have been achieved to date (Yu and Powles, 2014; Wang et al., 2017; Oliveira et al., 2018; Van Etten et al., 2020). It is believed that recurrent selection by low herbicide dosages plays a major role in the stacking of multiple small-effect alleles conferring NTSR (Délye, 2013), and the interactions among the resistance alleles may play an important role in the resistance level (Renton et al., 2011).

Although less often acknowledged in the literature, stresses caused by sub-lethal herbicide doses may play an important role in the evolution of NTSR (as reviewed by Dyer, 2018) by inducing systemic stress responses that lead to genetic and epigenetic changes upon which selection can act (Ram and Hadany, 2014; Hu et al., 2016; Kim et al., 2017). These epigenetic modifications driven by environmental cues during the plant life cycle can be inherited and remain stable for as long as the stressors remain (Cubas et al., 1999; Hsieh et al., 2016).

Identifying the underlying genetic basis of NTSR is a challenging task that takes time and resources. To date, several examples of these attempts are available in the literature, and scientists have been able to identify candidate genes efficiently (see discussion below). Further validation of candidate alleles via functional analysis are rare; however, these are the

ultimate approaches necessary to relate the genotype with the resistance phenotype.

High-throughput sequencing technologies, associated with the exponential cost reduction of these technologies, have enabled researchers to acquire massive amounts of data, not only for model species (e.g., *A. thaliana*) but also for non-model organisms, as is the case of *Lolium* spp. This enormous data quantity makes possible genome-wide interrogations of causal genetic features associated with traits of interest. Although such interrogations are common place in other disciplines, limited research has explored the underlying basis of NTSR in weed populations. Different methods have different benefits and drawbacks, and existing knowledge of the target organism will aid in the choice of the most appropriate approach to study NTSR.

Transcript expression quantification has been used in the field of weed science to investigate the mechanisms of NTSR. Prior information on the potential enzymes and herbicide metabolites involved in the NTSR are essential when low-throughput methods are adopted to study the resistance mechanisms (e.g., real-time quantitative polymerase chain reaction), as these approaches are very laborious and time consuming (Iwakami et al., 2014a,b; Guo et al., 2019). When limited information about the physiological and biochemical aspects of a resistance phenotype is available, high-throughput sequencing approaches (i.e., RNseq) may be a better option. Careful consideration of the experimental design plays an important role in the success of the RNA-seq analysis (Giacomini et al., 2018). Given the limited genomics resources currently available in most weed species, a *de novo* reference transcriptome assembly is the first step in a differential expression analysis (Gaines et al., 2014; Keith et al., 2017; Zhao et al., 2017). Another consideration when designing RNA-seq studies is the genetic background control of the experimental units, as it might determine the number of differentially expressed contigs identified (as reviewed by Giacomini et al., 2018). It is recommended that crosses be performed before final RNA extraction, so that researchers may take advantage of recombination and reduce the number of candidate genes. Following quantification of differentially expressed contigs, further analysis is necessary to identify candidate genes, and typically require a prior physiological and biochemical knowledge of the phenotype. By filtering contigs unlikely to be involved in pyroxsulam resistance in *Lolium* spp. (Duhoux et al., 2015), a list of differentially expressed genes was reduced from > 10,000 to four candidate genes. Similarly, Zhao et al. (2017) focused on the validation of 31 candidate genes from a pool of > 11,000 differentially expressed contigs in *Alopecurus aequalis*. Upon identification of candidate genes, functional analysis of the differentially expressed genes is necessary to confirm involvement in the mechanisms of resistance. Functional analysis can be achieved by performing knockout, knockdown, or upregulation of gene constructs in model plant organisms (however, see Mellado-Sánchez et al., 2020). Inherently, RNA-seq experiments are an exploratory approach, especially to design new hypotheses for a given phenotype, and should not be used as a stand-alone means to answer biological questions regarding NTSR.

Another strategy to identify NTSR is to look for signatures of selection in the weed genome. The idea behind this suite of techniques is to use population genomics approaches to identify loci under selection using a set of statistical tests. Because selection will shape the frequency of the alleles under selection, markers with unusual allele frequencies within and among populations may be compared using genetic markers. A number of approaches to acquire genetic markers have been used (Paris et al., 2010), with the bottom line to compare the distribution of marker data to a distribution of markers assumed to be under a neutral model of evolution. Although most types of markers may be used to perform such an analysis, single nucleotide polymorphisms (SNP's) have been preferred as thousands of genome wide markers may be acquired with next-generation sequencing instruments. Genome-wide analysis provides the benefit of discovering new loci involved in the resistance traits. Many software programs have been developed to associate genotype with phenotype (reviewed by Hoban et al., 2016); however, a recent review of the outlier analysis usage between 2010 and 2016 (Ahrens et al., 2018) indicated ARLEQUIN was the most commonly program used for this purpose (Excoffier et al., 2005).

Outlier approaches are prone to a number of biases (e.g., false positives, confounded effects due to population structure, spatial correlation; reviewed by Hoban et al. (2016), therefore combining multiple approaches are typically beneficial to validate candidate loci. Examples of these approaches have been limited in the weed science literature, however are not absent. Kreiner et al. (2019) evaluated genetic differentiation using 100-kb sliding windows between resistant and susceptible *A. tuberculatus*, and found evidence that regions containing the EPSPS coding region were highly differentiated and likely involved in the resistance phenotype. Van Etten et al. (2020) studied eight populations of glyphosate-resistant and -susceptible *Ipomoea purpurea* from the Southeastern and Midwest United States that did not exhibit TSR. These authors adopted SNP outlier approaches to survival and resistance level data using and identified 42 to 83 loci (depending on the approach utilized) potentially involved in the glyphosate resistance trait. Following an exome resequencing step and outlier analysis, the authors were able to identify five genomic regions under positive selection that contained enriched genes in the cytochrome P450, ABC transporters, glycosyltransferases, and GST families. Although glyphosate metabolism has rarely been involved in the resistance mechanisms, more research is needed to confirm the involvement of metabolizing enzymes in *I. purpurea*. Similar approaches could be successful if implemented to uncover NTSR mechanisms in *Lolium* spp.

Genome-wide association studies (GWAS) rely on statistical models to find correlations between an observed phenotype and the genotype (reviewed by Leon et al., 2020). With high-throughput sequencing technologies, thousands to millions of SNP's may be identified and can be used for association studies. These associations may be prone to high false positive rates if the statistical method chosen is not adequate to correct for confounding factors inherent from the study populations, such as population structure and unequal relatedness (Zhang et al., 2010). Several methods have been established to reduce

false positive errors (Segura et al., 2012; Liu X. et al., 2016), and GWAS has been successfully implemented to identify the underlying genetic basis of traits in plants (Huang and Han, 2014). Although the availability of a reference genome may assist in the functional analysis of genomic regions, GWAS may be performed *de novo* (without the aid of a reference genome) (Voichek and Weigel, 2020). Limited weed science literature is available where GWAS was used to detect NTSR. Kreiner et al. (2020) conducted a GWAS in glyphosate-resistant *A. tuberculatus* that exhibited increased EPSPS duplication in the majority of the populations tested and, as expected, found that the genomic regions containing the EPSPS coding sequences were related to the resistance phenotype. The authors also found > 100 genes across the weed genome involved in the glyphosate resistance, and were identified as involved in stress-response and NTSR.

Regardless of the method employed to identify SNP's throughout the weed genome, it is crucial that a thorough analysis is performed before the start of any experiment. An important consideration is the genome size. Analysis that rely on genome-wide SNP's are typically performed with the assistance of restriction enzymes, which vary in the frequency that they cleave their recognition site. If a rate cutter restriction enzyme is chosen for a genome the size of *Lolium* spp. (approximately 2 Gb), it is very unlikely that the identified SNP's will be physically linked (i.e., in linkage disequilibrium) with the causal mutation. Another consideration is the approach to construct the plant population that will be used for the analysis (reviewed by Morrell et al., 2012), which will also determine the likelihood of success in determining the genomic regions involved in the NTSR.

CONCLUSION AND FUTURE DIRECTIONS

Lolium spp. exhibit an astonishing potential to evolve herbicide resistance, likely due to its high genetic diversity and ability to exchange genetic material due to gene flow (Matzrafi et al., in press). *Lolium* spp. populations around the world have evolved NTSR to many herbicides. Because NTSR may be non-specific, populations may exhibit unknown herbicide resistance patterns, as resistance occurs to herbicides to which populations have never been exposed. NTSR poses a challenge to sustainable agricultural production systems, and is an ongoing issue that needs a collaborative approach to be minimized.

Non-target-site resistance research has elucidated fascinating aspects of how *Lolium* spp. evolve herbicide resistance, and adopted creative approaches to uncover the details of how plants manage to survive lethal doses of herbicides. Most of the efforts have been to describe the physiological and biochemical alterations that take place at the plant and cellular level (e.g., reduced herbicide translocation, herbicide metabolism). The underlying genetic bases of the phenotypes remain largely unknown. For instance, it is currently unknown which genes are involved in the vacuolar sequestration of paraquat in *L. multiflorum*. It is also unknown how these herbicide resistance genes arise in the populations (see modes of convergent adaptation in Lee and Coop, 2017). Information on the underlying genetic basis of the resistance mechanism has not only basic, but also applied applications. For instance, genetic markers may be developed to identify seed lots contaminated with herbicide resistant *Lolium* spp. seed, or field diagnostics to quickly identify herbicide susceptibility before growers treat an infested field. Policymakers may use information on how resistance genes arise in the population (i.e., gene flow, standing genetic variation, or new mutations; see Lee and Coop, 2017) to design regulations to prevent gene flow. Advancing our knowledge on NTSR resistance in *Lolium* spp. will require efforts of multidisciplinary teams that will likely include weed scientists, population geneticists, plant physiologists, biochemists, stakeholders, and funding agencies.

AUTHOR CONTRIBUTIONS

AKS, LB, CM-S, and CB wrote the manuscript. CM-S and CB conceptuaized the project. CB supervised the project. All authors contributed to the article and approved the submitted version.

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Target-Site and Non-target-Site Resistance Mechanisms Confer Multiple and Cross- Resistance to ALS and ACCase Inhibiting Herbicides in *Lolium rigidum* From Spain

Joel Torra[1], José María Montull[1], Andreu Taberner[1], Nawaporn Onkokesung[2], Neil Boonham[2] and Robert Edwards[2]*

[1] Department d'Hortofructicultura, Botànica i Jardineria, Agrotecnio, Universitat de Lleida, Lleida, Spain, [2] Agriculture, School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom

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***Correspondence:**
Joel Torra
joel.torra@udl.cat

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Lolium rigidum is one the worst herbicide resistant (HR) weeds worldwide due to its proneness to evolve multiple and cross resistance to several sites of action (SoA). In winter cereals crops in Spain, resistance to acetolactate synthase (ALS)- and acetyl-CoA carboxylase (ACCase)-inhibiting herbicides has become widespread, with farmers having to rely on pre-emergence herbicides over the last two decades to maintain weed control. Recently, lack of control with very long-chain fatty acid synthesis (VLCFAS)-inhibiting herbicides has been reported in HR populations that are difficult to manage by chemical means. In this study, three Spanish populations of *L. rigidum* from winter cereals were confirmed as being resistant to ALS- and ACCase-inhibiting herbicides, with broad-ranging resistance toward the different chemistries tested. In addition, reduced sensitivity to photosystem II-, VLCFAS-, and phytoene desaturase-inhibiting herbicides were confirmed across the three populations. Resistance to ACCase-inhibiting herbicides was associated with point mutations in positions Trp-2027 and Asp-2078 of the enzyme conferring target site resistance (TSR), while none were detected in the ALS enzyme. Additionally, HR populations contained enhanced amounts of an ortholog of the glutathione transferase phi (F) class 1 (GSTF1) protein, a functional biomarker of non-target-site resistance (NTSR), as confirmed by enzyme-linked immunosorbent assays. Further evidence of NTSR was obtained in dose-response experiments with prosulfocarb applied post-emergence, following pre-treatment with the cytochrome P450 monooxygenase inhibitor malathion, which partially reversed resistance. This study confirms the evolution of multiple and cross resistance to ALS- and ACCase inhibiting herbicides in *L. rigidum* from Spain by mechanisms consistent with the presence of both TSR and NTSR. Moreover, the results suggest that

NTSR, probably by means of enhanced metabolism involving more than one detoxifying enzyme family, confers cross resistance to other SoA. The study further demonstrates the urgent need to monitor and prevent the further evolution of herbicide resistance in *L. rigidum* in Mediterranean areas.

Keywords: glutathione-s-transferase, ACCase inhibitor, ALS inhibitor, cytochrome P450 monooxygenase, enhanced metabolism, photosystem II inhibitor, thiocarbamate herbicide, very-long-chain fatty acids biosynthesis inhibitor

INTRODUCTION

Lolium rigidum (Gaud.) is a genetically diverse, cross-pollinating and globally distributed weed species, that has evolved resistance to herbicides acting on many sites of action (SoA) around the world (Yu and Powles, 2014). To date, *L. rigidum* has evolved resistance to 14 different herbicide SoA around the globe (Heap, 2020). In Europe, resistance to acetyl CoA carboxylase (ACCase), acetolactate synthase (ALS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), glutamine synthase, and protoporphyrinogen oxidase (PPO) inhibiting herbicides has been reported in *L. rigidum* associated with winter cereals, vineyards and orchards in the southern part of the continent (De Prado et al., 2005; Cirujeda and Taberner, 2010; Kaloumenos et al., 2012; Atanackovic et al., 2015; Fernandez-Moreno et al., 2017; Fernández-Moreno et al., 2017; Heap, 2020). As such, multiple, or cross-resistance to several SoA is now widespread in southern Europe.

In rainfed cereals from Spain, the first case of a herbicide resistant (HR) *L. rigidum* Gaud. population was reported in the north-eastern region, with resistance reported to chlortoluron (PSII inhibiting herbicide) and diclofop-methyl (ACCase inhibiting herbicide) in 1995 (Taberner et al., 1995). Failures in the control of *L. rigidum* with chlorsulfuron (ALS inhibiting herbicide) were subsequently reported in the river Duero region in Castilla-León (de la Carrera et al., 1999). ACCase, ALS, and PSII inhibitors resistance is now widespread throughout the Spanish winter cereal cropping system (Loureiro et al., 2017), with 75% of *L. rigidum* populations in Catalonia exhibiting HR to these SoA (Loureiro et al., 2010, 2017). The resistance problem is becoming even more complex, with HR reported toward other graminicides used in canola, including clethodim and fluazifop-butyl (Cirujeda and Taberner, 2003).

In response to HR toward ACCase, PSII, and ALS inhibiting, since 2000 there has been an increased reliance on pre-emergence soil-applied herbicides such as prosulfocarb from the thiocarbamate family (Group 15 HRAC/WSSA, inhibition of fatty acid elongase) and less often trifluralin from the dinitroaniline family (Group 3, inhibition of microtubule assembly). Unfortunately, trifluralin has been banned in the European Union since 2009, primarily due to toxicity in aquatic environments (Commission Decision 2010/455/EU; 26 June 2010). This has placed greater pressure on herbicides from groups 15, such as oxyacetamides (flufenacet) or chloroacetamides (metazachlor) and group 3 (propyzamide). These are the only available MoA, excluding non-selective herbicides, still available to control problematic *L. rigidum* in Spanish winter cereals and rotational crops.

Resistance to ALS and ACCase inhibiting herbicides can be caused by a mutation in the genes encoding the respective proteins, which is termed target-site resistance (TSR). The mutations at specific positions on ALS or ACCase enzyme commonly confer resistance to specific herbicide chemistries. In *L. rigidum*, amino acid substitutions at positions Pro197 and Trp574 (among three other positions) in the ALS enzyme are the common mutations associated with the resistance to sulfonylurea (SU) ALS-inhibiting herbicides, while the second position to imidazolinone (IMI) too (Yu et al., 2008; Tranel et al., 2020). The amino acid substitutions at positions Ile1781, Trp2027, Ile2041, and Asp-2078 (among two other positions) of ACCase, have been frequently identified to confer resistance to aryloxyphenoxypropionates (APP) and cyclohexanediones (CHD) herbicides (Kaundun, 2014; Murphy and Tranel, 2019). Besides TSR, this species can also develop non-target-site resistance (NTSR) to multiple herbicide chemistries through various mechanisms including increased herbicide detoxification, also referred to as enhanced metabolic resistance (EMR). EMR has been recorded in *L. rigidum* both for ACCase and ALS inhibiting herbicides (Gaines et al., 2020). Enhanced herbicide metabolism is associated with the increased expression of detoxifying enzymes, including cytochrome P450 monooxygenases (CYP450) and glutathione *S*-transferases (GSTs) (Ghanizadeh and Harrington, 2017). In EMR *L. rigidum* populations, the CYP450 family has been identified as one of the important enzyme family conferring resistance to ACCase and ALS inhibiting herbicides (Gaines et al., 2020).

GSTs are known to detoxify herbicides through catalyzing their conjugation with glutathione (GSH) (Cummins et al., 2013; Gaines et al., 2020). In *Alopecurus myosuroides*, the constitutive level of a glutathione transferase phi (F) class 1 protein (*AmGSTF1*) was significantly higher in multiple herbicide resistance populations than in sensitive populations (Comont et al., 2020). The biochemistry of *AmGSTF1* and its transgenic expression in *Arabidopsis thaliana* showed that this protein has a functional role in regulating NTSR that extends beyond the glutathione conjugation of herbicides (Cummins et al., 1999, 2013). Furthermore, the increased expression of the orthologous transcript and protein *LrGSTF1* in *L. rigidum* populations is also associated with NTSR (Cummins et al., 2013; Busi et al., 2018). Together, this information indicates that the level of the *AmGSTF1* ortholog, *LrGSTF1*, could be used as a biomarker to identify the existence of NTSR in *L. rigidum* populations.

In HR *L. rigidum* populations from rainfed cereals in Spain, the resistance mechanisms to ACCase inhibiting herbicides were previously studied (De Prado et al., 2005), while those conferring resistance to ALS have not been reported. Moreover, cross- and multiple herbicide resistance across different chemistries by EMR and the enzymes involved have not been reported. Furthermore, the first cases of failures in *L. rigidum* control with other herbicide SoA apart from ALS or ACCase inhibitors were reported in Catalonia in 2010 (Comité para la Prevención de Resistencias a Herbicidas [CPRH], 2015). Therefore, the identification of the associated herbicide resistance mechanisms is now required for tailoring the weed management of multiple HR *L. rigidum* populations in winter cereals in Spain.

In this study, the potential for TSR and NTSR mechanisms to evolve toward ALS- and ACCase inhibiting herbicides was investigated in three populations from Spain. Also, resistance to thiocarbamate herbicides was characterized in whole plant studies. Additionally, we report on the response of these populations to PSII-inhibiting herbicides, fatty acid elongase inhibiting herbicides, and fatty acid synthase plus carotenoid biosynthesis inhibiting herbicides.

MATERIALS AND METHODS

Plant Material

Lolium rigidum populations used in this study came from winter cereal fields where lack of control after prosulfocarb treatments was reported. All the fields were located in North-Eastern Spain, with prosulfocarb being the only herbicide applied over several years (≥5 years). This herbicide is usually applied when ACCase, ALS, and PSII inhibiting herbicides do not provide sufficient efficacy. Populations, both resistant (R) and susceptible (S), were collected during 2014 and 2015 (**Table 1**). The population considered as S had never been exposed to herbicides.

Seeds were incubated at 40°C for 3 weeks to break seed dormancy and then stratified at 4°C for 7 days to promote and synchronize germination. After 7 days, germination plates were moved to the growth cabinet (16/8 h light, 18°C) for 1 week before transplanting four germinated seedlings per pot.

Herbicide Screening

In this experiment, 10 seeds per pot were sown in 7 × 7 × 9 cm pots filled with a mixture of soil, perlite and peat (4/1/2 v/v/v). Pots were placed in a greenhouse located in the University of Lleida, north-eastern Spain (41°37′N, 0°38′E) and kept with a light regime of 14 h at 25°C, provided by supplementary lighting, and 10 night hours at a minimum temperature of 10°C. Irrigation was at demand, daily, through a shower system without water reuse, with fertilization as required. One week after sowing PRE herbicides were applied at the BBCH stage of 00-09 (Zadoks et al., 1974). To test herbicides in POST, after seedling emergence the plants per pot were thinned to four, and then were sprayed at the BBCH stages of 11-12 or 13-21 (Zadoks et al., 1974; **Table 2**). Herbicide screening was conducted as described in **Table 2**.

The active ingredient florasulam in Broadway predominately provides broad-leaf weed control. Therefore, for the purposes of this screening, the efficacy of the other active ingredient in Broadway, pyroxsulam, was evaluated against *L. rigidum*. In Herold, the active ingredient flufenacet has the main activity against *L. rigidum*, as compared with diflufenican, and was the subject of this study. Due to the large number of herbicides/populations to be tested and seed availability, only three rates per herbicide were used (**Table 2**). Herbicides were applied using a precision bench sprayer delivering 200 L ha^{-1}, at a pressure of 200 kPa and equipped with two Hardi Flat fan nozzles. 28 days after treatment plants were harvested (above ground) and fresh weight measured. Experiments were repeated twice.

TABLE 1 | *Lolium rigidum* populations from Spanish winter cereal fields, location, year of seed collection, and potential resistance profile.

Biotype	Locality	Year of seed collection	Profile
ES-MHR 1	Calonge de Segarra	2015	Multiple Herbicide Resistant
ES-MHR 2	Calonge de Segarra	2015	Multiple Herbicide Resistant
ES-MHR 3	Calaf	2014	Multiple Herbicide Resistant
ES-S 1	Ballobar	2014	Susceptible

Rapid Diagnosis of Specific Mutations in ACCase- and ALS Genes

The mutations in ACCase or ALS enzyme were analyzed by Loop-Mediated Isothermal Amplification (LAMP) assay. The specific probes to detect single nucleotide polymorphisms (SNPs) at specific position in ACCase (GenBank accession AJ310767) or ALS (AJ437300) enzymes were designed using DNA sequences from *A. myosuroides*. Leaf samples of three putative HR *L. rigidum* (ES-resistant 1 to 3) and susceptible (ES-susceptible 1) populations (BBCH 13-15) were collected from plants grown as described previously, with five pots per population. Five leaf blades (∼0.5 cm/leaf blade) from five individual plants were pooled together for testing the mutations. For each population, three biological replicates were assayed per probe. 750 μL of alkaline-polyethylene glycol solution (pH 13.4) was added to leaf samples and the samples were shaken for 30 s to extract DNA. 5 μL of DNA samples were added to the reaction contained 15 μL Isothermal MasterMix (ISO-001, OptiGene) and 5 μL of specific probes (OptiGene)for each type of mutation in ALS (Trp 574) or ACCase (Ile-1781, Trp-2027, Ile-2041, and Asp-2078) protein. The LAMP reactions were performed with a Genie III (OptiGene) machine. The reactions were activated at 65°C for 30 min followed by an isothermal step at 95°C for 2 min and annealing from 40 to 70°C. The SNP at each position was identified by the shifting of melting temperature of the product compared to those of wild type form using synthetic DNA constructs (g-block) (**Supplementary Table 1**). The tests were done using pooled samples. As such, the mutation frequency within the population was not determined.

TABLE 2 | Trade name, company, active ingredients, and sites of action of herbicides tested on the *Lolium rigidum* populations.

Trade Name	Company	Active ingredients	Site of Actions	BBCH	Rates (g ha^{-1})
Atlantis	Bayer CropScience	Mesosulfuron ++ iodosulfuron	ALS inhibitor	13–21	4+1, 8+2, 15+3
Broadway Star	Dow AgroSciences	Pyroxsulam + florasulam	ALS inhibitor	13-21	9+2, 19+4, 38+8
Iloxan	Bayer CropScience	Diclofop-methyl	ACCase inhibitor	13-21	720, 360, 180
Select	FMC	CLETHODIM	ACCase inhibitor	13-21	24, 48, 96
Clortolurex	Adama Agriculture	Chlortoluron	PS II inhibitor	11-12	375, 750, 1500
Herold	Bayer CropScience	Flufenacet + diflufenican	Groups 15+12	00-09	60+30, 120+60, 240+120
Auros	Syngenta Agro	Prosulfocarb	Group 15	00-09	1,000, 2,000, 4,000

All companies were located in Spain. 15, inhibitors of the fatty acid synthase; 12, carotenoid biosynthesis inhibitors; group 15, fatty acid elongase inhibitor. BBCH: growth stage at which herbicides were applied. Three doses were applied per herbicide; being the highest maximum registered dose in Spain.

Non-target-Site Resistance Testing: Analysis of a Biomarker Protein Using Enzyme-Linked Immunosorbent Assay (ELISA)

The level of LrGSTF1 protein was analyzed in total protein extract from leaf tissue. Leaf tissue from five individual plants (BBCH 13–15) were pooled into one biological replicate with five biological replicates used for quantification. Total protein was extracted from frozen ∼100 mg (fresh mass) of leaf tissue by grinding in liquid Nitrogen before adding 900 μL extraction buffer (100 mM Tris–HCl, 150 nM NaCl, 5 mM EDTA, 5% glycerol, 2% PVPP, and 10 mM DTT; pH 7.5). The leaf suspensions were incubated on ice for 10 min before centrifugation at 12,000 × *g*, 4°C for 30 min. The supernatants were collected and recentrifuged for 15 min. The total protein concentration of clear supernatants was quantified by the Bradford assay following the manufacturer's protocol (Bio-Rad Laboratories, United Kingdom). The concentration of the polypeptides recognized by the anti- *AmGSTF1*-serum from *L. rigidum* protein samples was determined using ELISA (Davies et al., 2020). The concentration of the homolog LrGSTF1 protein in *L. rigidum* samples (five biological replicates per population) were calculated using a standard curve prepared using recombinant *AmGSTF1* protein (non-linear regression four parameters logistic analysis; Graphpad PRISM v.8.2).

P450: Herbicide Synergism of Malathion in Whole Plant Studies

To study the potential role of CYP450 in NTSR by enhanced metabolism in these HR *L. rigidum* populations, a CYP450 inhibitor was applied in post-emergence to assess its synergism with the herbicide prosulfocarb. All populations were sprayed at the BBCH stage of 13-21 (Zadoks et al., 1974) with prosulfocarb (Auros, Syngenta Agro, 80%) at 0, 400, 800, 1600, 3,200 (field rate), and 6,400 g ha^{-1}. One hour before herbicide treatment, all populations were sprayed with either 0 or 1,000 g a.i. ha^{-1} of the organophosphate insecticide malathion {[(dimethoxyphosphinothioyl)-thio] butanedioic acid diethyl ester}. Previous research has shown that 1,000 g a.i. ha^{-1} is around the maximum dose of malathion that can be used without adverse effects in *L. rigidum* (Christopher et al., 1994; Preston et al., 1996; Yu et al., 2009). A total of four replicates were included at each dose, with non-treated plants used as controls and prosulfocarb applied as described above. Percentage of survival was estimated in each case, and plants were harvested (above ground) and the fresh weight measured. Experiments were repeated twice.

It is important to note that thiocarbamates such as prosulfocarb are initially CYP450-mediated oxidized to the sulfoxide, the active form of the herbicide (Fuerst, 1987). To assess the role of CYP450 in activating this herbicide, all populations were applied with a pre-emergence treatment after a pre-treatment with malathion in a second run of experiments. Populations were sprayed at the BBCH stage of 00-10 (Zadoks et al., 1974) with prosulfocarb at 0, 1,200, 2,400, and 4,000 g ha^{-1} (maximum field rate). One hour before herbicide treatments, all populations were treated with either 0 or 1,000 g a.i. ha^{-1} of malathion. Experimental design and applications were as described previously. Only survival was estimated. Population ES-resistant 3 was not included in this study due to limited seed availability.

Statistical Analysis

Sensitivity was assessed for each *L. rigidum* population at each herbicide treatment by expressing the percentage of reduction in measured fresh biomass as compared to that determined in the untreated controls. The requirement of homogeneity of variance was checked by visual inspection of the residual plots and residuals were analyzed using the Shapiro–Wilk Test. When required, data were previously transformed. A two-way ANOVA was performed on untransformed data for each herbicide screen (ACCase inhibitors, ALS inhibitors, chlortoluron, prosulfocarb, and flufenacet plus diflufenican). Population and herbicide dose were used as factors and replicates used as an error term (block). Tukey's honest significant difference (HSD) tests were performed to compare the percentage control of suspected resistant populations relative to the known HS plants for each species.

Differences in amounts of the AmGSTF1 were compared among populations using one-way ANOVAs followed by Tukey's HSD test using SPSS v.24 software (IBM, Chicago, IL, United States). The assumption of homogeneity of variance for one-way was tested by Levene's test. As the data did not fit the normal distribution, it was transformed in to log10 before doing ANOVA.

Data from dose-response experiments with a pre-treatment of malathion in POST were analyzed using a non-linear regression model. The herbicide rates required for 50% survival reduction (LD_{50}), or 50% growth reduction in fresh weight of plants (GR_{50}) and for 90% survival reduction (LD_{90}), or 90% growth reduction (GR_{90}) were calculated with the use of a four parameter logistic curve (Seefedlt et al., 1995), of the type:

$$y = c + \frac{d - c}{1 + EXP\left[b\left(log(x) - log\left(LD50\ or\ GR50\right)\right)\right]}$$

where c = the lower limit set to 0, d = the upper limit set to 100, and b = the slope at the LD_{50} or GR_{50}. In this regression equation, the herbicide rate (g a.i. ha^{-1}) was the independent variable (x) and the survival (%) or plants' fresh weight expressed as percentage of the untreated control were the dependent variables (y). The resistance index (RI) was computed as $RI_{50} = LD_{50}$ or $GR_{50}(R)/LD_{50}$ or $GR_{50}(S)$ or as $RI_{90} = LD_{90}$ or $GR_{90}(R)/LD_{90}$ or $GR_{90}(S)$, both for survival or fresh weight reduction. Repetitions from the dose-response experiments were pooled due to lack of statistical differences between them. Non-linear regressions were carried out with the use of Sigmaplot 11.00 (Systat Software, 2007).

Survival data from the experiment with a pre-treatment of malathion in POST was analyzed with a three-way ANOVA. Population, inhibitor and herbicide dose were used as factors and replicate used as an error term (block). Tukey's honest significant difference (HSD) tests were performed to compare treatment means.

RESULTS

Herbicide Screening

All three *L. rigidum* populations used in this study were poorly controlled by both ACCase- and ALS-inhibiting herbicides. Statistical comparison of these three populations against S plants showed that they had a significantly lower percentage reduction in fresh biomass at all herbicide rates tested (**Figure 1**). For the ACCase inhibitors clethodim and diclofop, the fresh biomass reductions were lower than 60% in most cases, except for the

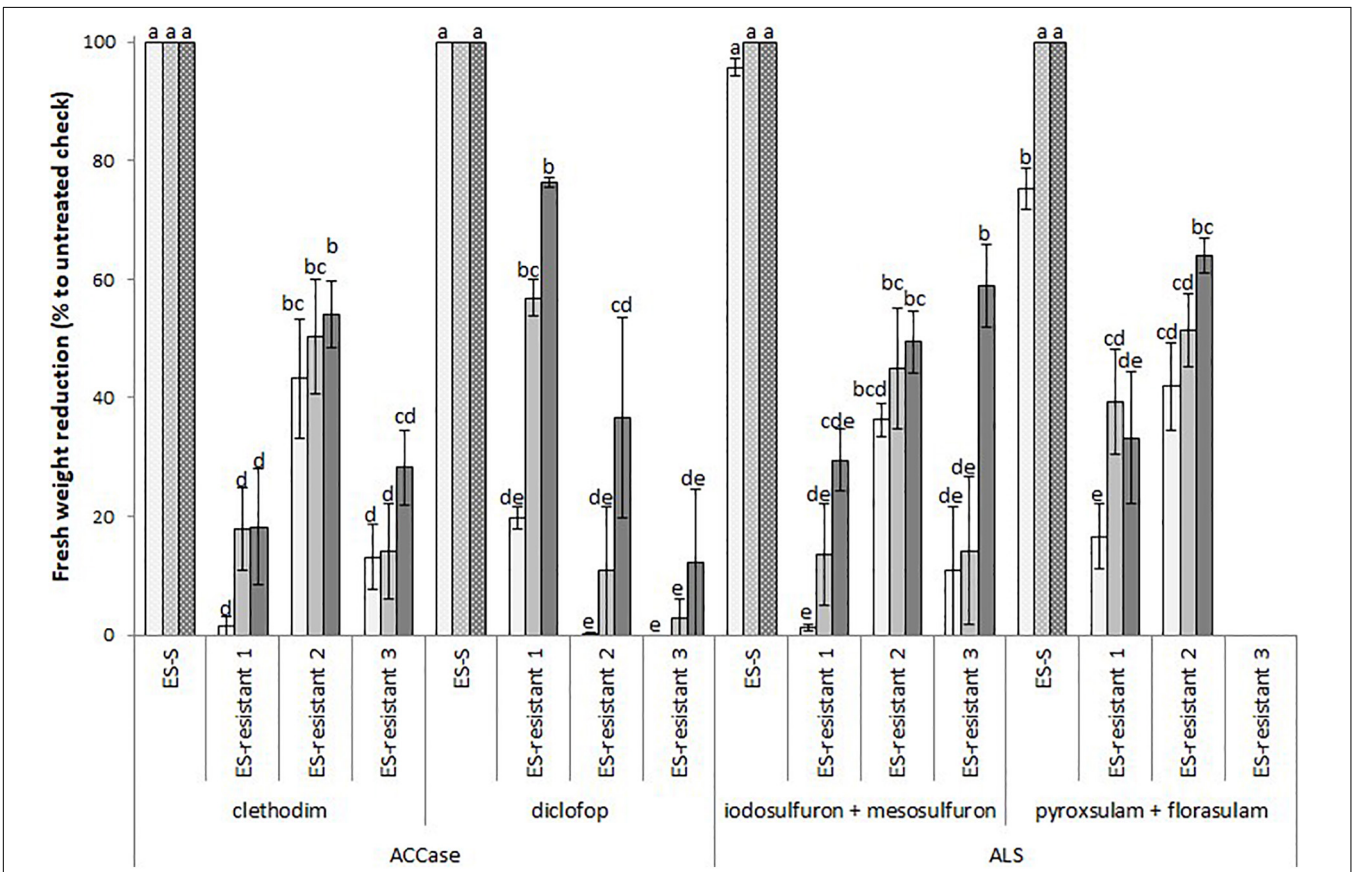

FIGURE 1 | Mean reduction in foliage fresh biomass relative to untreated control for multiple HR populations of *Lolium rigidum* to ACCase (group 1, HRAC/WSSA) and ALS inhibitors (group 2). Populations ES-resistant 1 to 3 compared to a known sensitive population (ES-S). Treated with four herbicides at three rates each (columns with different gray intensity from lowest to highest rate): two ACCase inhibitors, clethodim (24, 48, and 96 g ha^{-1}) and diclofop (180, 360, and 720 g ha^{-1}), and two ALS inhibitors, mesosulfuron (3.8, 7.5, and 15 g ha^{-1}) plus iodosulfuron (0.8, 1.5, and 3 g ha^{-1}), and pyroxsulam (9.4, 18.8, and 37.5 g ha^{-1}) plus florasulam (1.9, 3.8, and 7.5 g ha^{-1}). Error bars are standard error of the mean. N.D., not determined.

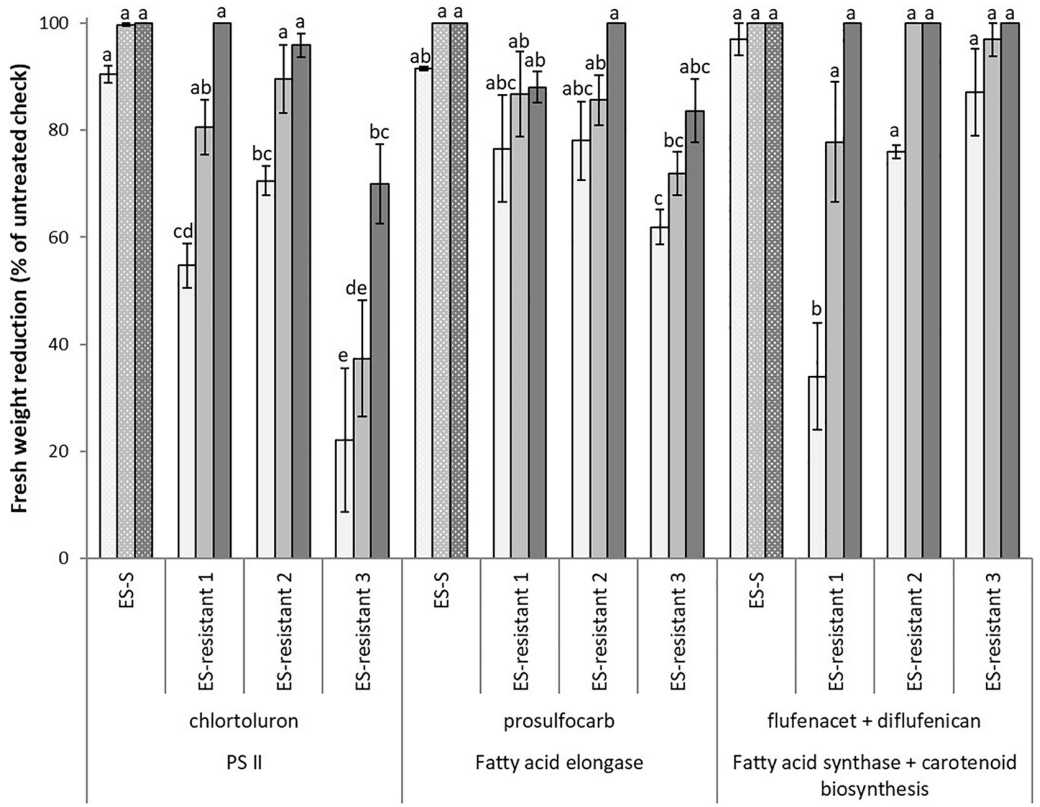

FIGURE 2 | Mean reduction in foliage fresh biomass relative to untreated control for multiple HR populations of *Lolium rigidum*. Populations ES-resistant 1 to 3 compared to a known sensitive population (ES-S). Treated with three herbicides at three rates each (columns with different gray intensity from lowest to highest rate): one PS II inhibitor (group 5, HRAC/WSSA), chlortoluron (750, 1,500, and 3,000 g ha^{-1}), one fatty acid elongase inhibitor, prosulfocarb (group 15, thiocarbamates) (1,000, 2,000, and 4,000 g ha^{-1}), and one fatty acid synthase inhibitor, flufenacet (group 15, oxyacetamides) (60, 120, and 240 g ha^{-1}) plus one carotenoid synthesis inhibitor, diflufenican (group 12) (30, 60, and 120 g ha^{-1}). Error bars are standard error of the mean.

ES-resistant 1 population that showed ∼80% biomass reduction when treated with the highest dose of diclofop. Similarly, the reduction in fresh biomass compared to the S population did not extend beyond 60% for the ALS inhibitors iodosulfuron + mesosulfuron and pyroxsulam + florasulam treatments. An exception was the treatment with the highest dose of pyroxsulam + florasulam in the ES-resistant 2 population (64%). No data was available for the ES-resistant 3 populations for the mixture of ALS-inhibitors pyroxsulam + florasulam. Together, these results suggested that the three resistant populations of *L. rigidum* showed enhanced tolerance to multiple chemistries of ACCase- and ALS-inhibiting herbicides.

Besides enhanced resistance to ALS- and ACCase-inhibiting herbicides, poor controls in terms of fresh weight reduction (22–89%) were observed in the three HR populations when treated with the PS II inhibitor chlortoluron at 0.25 and 0.5x rates (**Figure 2**). While at field recommended rate (1,500 g ha^{-1}), only ES-resistant 3 population showed a lower percentage of fresh weight reduction (70%) compared to the S population.

Apart from the resistance to post-emerging herbicides, the three HR populations showed a lower percentage of fresh biomass reduction at all rates of prosulfocarb treatments (62–88%), except for the highest rate tested (4,000 g ha^{-1}) in the ES-resistant 2 population (**Figure 2**). Finally, for the formulated mixture flufenacet plus diflufenican, no significant differences were found among the populations, though reduced susceptibility was observed at the lowest rate in the ES-resistant 1 and 2 populations (34 and 76%), and also at the intermediate rate (120 + 60 g ha^{-1}) in ES-resistant 1 (78%) (**Figure 2**).

Taken together, our results confirmed that all three HR *L. rigidum* populations studied showed very high levels of increased tolerance to ACCase- and ALS-inhibiting herbicides, as well as moderate tolerance toward PS II and Fatty acid elongase inhibitors (**Table 3**). Overall, the ES-resistant 3 population, showed the highest levels of increased tolerance (cross-resistance) to these four MoAs. It is noteworthy that none of the researched populations showed increased tolerance to the herbicide mixture flufenacet + diflufenican.

Rapid Diagnosis of Specific Mutations in ACCase- and ALS Genes

The high level of resistance to specific chemistries of ALS or ACCase-inhibiting herbicides including mesosulfuron+iodosulfuron (SU), diclofop (APP) and clethodim (CHD) in *L. rigidum* are known to be caused by the mutations at

TABLE 3 | Summary of the resistance profiles of one susceptible and three multiple HR populations of *Lolium rigidum* from Spain to five SoA.

	ALS		ACCase		PS II	F. a. elongase	F. a. synthase+carotenoid
Population	**Iodosulfuron+mesosulfuron**	**Pyroxsulam+florasulam**	**diclofop**	**clethodim**	**chlortoluron**	**prosulfocarb**	**Flufenacet+diflufenican**
ES-S	S	S	S	S	S	S	S
ES-R 1	R	R	R	R	MR	MR	MR
ES-R 2	R	R	R	R	MR	MR	LR
ES-R 3	R	–	R	R	MR	R	LR

F.a., fatty acid; S, susceptible; LR, low resistant (mean efficacy below 90% only at the lowest rate); MR, moderate resistant (mean efficacies below 90% at low and medium rates); R, resistant (mean efficacies below 80% at the three rates).

specific position in ALS or ACCase enzymes. To assess whether these specific mutations existed in the R populations, we utilized Loop-Mediated Isothermal Amplification (LAMP) as a novel rapid diagnosis technique. Although the LAMP assay will not provide information on the specific amino acid substitution, this technology will detect a SNP in the specified position in ALS or ACCase genes within 45 min. Hence, LAMP assay can be used as a tool to monitor the emergence of SNPs.

Based on the resistance profiles, we selectively tested specific SNPs in ALS (Pro-197 and Trp-574) and ACCase (Ile-1781, Trp-2027, Ile-2041, and Asp-2078) that have been previous reported to confer resistance to SU, APP, and CHD herbicides in *L. rigidum* (Yu et al., 2007, 2008). All plants tested from ES-resistant 1 and 2 populations possessed mutations at Trp-2027 and Asp-2078 positions (but not in Ile-1781 or Ile-2041) of the ACCase gene. However, no mutations of the ACCase gene at these selected positions were found in the ES-resistant 3 population (**Table 4**). It was interesting that the mutation at Asp-2078 has been reported to be a specific mutation that confers resistance to clethodim (CHD) in *L. rigidum* (Yu et al., 2007). Furthermore, the mutation at Trp-2027 has been reported to confer cross-resistance between APP and CHD herbicides (Yu et al., 2007). Together, these results suggest that the mutations in the ACCase enzyme might contribute to the high resistance to APP and CHD herbicides in ES-resistant 1 and 2 populations. Whereas, tolerance to ACCase inhibiting herbicides was more likely due to NTSR in the ES-resistant 3 population.

When *L. rigidum* populations were subjected for detecting the mutations at Pro-197 and Trp-574 in ALS genes, no plants from the three HR *L. rigidum* populations possessed mutations at Trp-574 position (**Table 4**), which is known to confer resistance to SU and IMI. However, we observed low and inconsistent signal which could lead to the false interpretation when the primers for detecting the SNP at Pro-197 position was used. Therefore, we excluded the results of Pro-197 from this study. It is noteworthy that the mutation at Pro-197 is known to be associated with resistance to SU such as mesosulfuron + iodosulfuron (Yu et al., 2008; Tranel et al., 2020). Although we cannot confirm the existent of Pro-197 in the R populations, it is possible, though unlikely based on reported incidence, that other mutations (Ala122, Ala205, and Asp376) could contribute to the observed resistance to SU (Murphy and Tranel, 2019; Tranel et al., 2020). Additionally, there is no report of a specific mutation in the ALS gene that confers resistance to pyroxsulam, a triazolopyrimidine (TRP), in *L. rigidum*.

TABLE 4 | The mutations in ALS or ACCase genes in Spanish *Lolium rigidum* populations, one susceptible and three putative multiple herbicide resistant.

	ALS		ACCase			
Populations	**Trp-574**	**Pro-197**	**Ile-1781**	**Trp-2027**	**Ile-2041**	**Asp-2078**
ES-sensitive 1	N.D.	N.R.	N.D.	N.D.	N.D.	N.D.
ES-resistant 1	N.D.	N.R.	N.D.	Mutation	N.D.	Mutation
ES-resistant 2	N.D.	N.R.	N.D.	Mutation	N.D.	Mutation
ES-resistant 3	N.D.	N.R.	N.D.	N.D.	N.D.	N.D.

N.D., not detected; N.R., not reported.
Three biological replicates were assay for each mutation.

Non-target-Site Resistance Mechanism Testing

GSTF1: Enzyme-Linked Immunosorbent Assay (ELISA)

To explore the possibility that NTSR could contribute to resistance to ACCase- and ALS-inhibiting herbicides and confer cross-resistance to other MoAs, the presence of the Lolium ortholog of *AmGSTF1*, a well-characterized biomarker of this type of resistance in wild grasses (Cummins et al., 2013), was assessed. Western blotting analysis showed that antisera raised to *AmGSTF1* detected a polypeptide typical of the respective GST subunit in all *L. rigidum* populations studied. The concentrations of the respective immunoreactive polypeptides were then determined by ELISA. The levels of AmGSTF1 immunoreactive polypeptides were significantly higher in the HR *L. rigidum* populations than in the S population (F = 5.998, P = 0.007). Irrespective of the presence of mutations in the ACCase or ALS genes, all HR populations showed enhanced levels of the AmGSTF1 ortholog (**Figure 3**). Moreover, the ES-resistant 3 population, which did not show mutation in the ACCase gene, had higher levels of the *AmGSTF1* ortholog protein together with ES-resistant 2 population, compared to the S one.

CYP450: Herbicide Synergism of Malathion in Whole Plant Studies

The CYP450 contains some of the most important detoxification enzymes that confer resistance to multiple herbicides in *L. rigidum*. To assess the involvement of CYP450, the resistance to herbicides following a pre-treatment with malathion

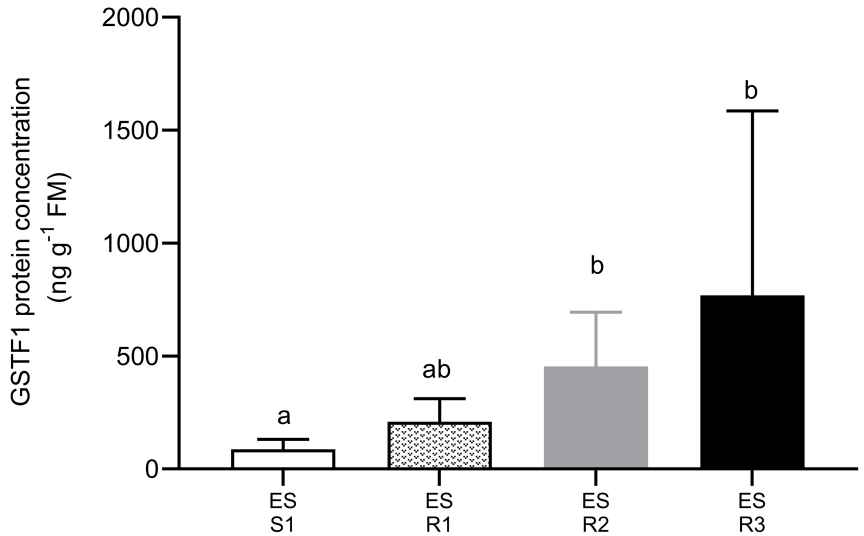

FIGURE 3 | The content of the ortholog protein of *AmGSTF1* in a susceptible and three multiple HR populations of *Lolium rigidum*. Ortholog protein abundance was quantified by ELISA. Each bar represented an average (mean ± SD, $n = 5$) of protein abundance in each population. Different letters indicate significant differences among population ($P \leq 0.05$).

TABLE 5 | Equation parameters of the log-logistic models used to estimate dose-response regression curves (% Survival and % fresh weight of untreated control) in susceptible (S) and potential prosulfocarb resistant *Lolium rigidum* populations (ES-R 1 to 3) for prosulfocarb with (+ malathion) or without (− malathion) pre-application of malathion at 1,000 g/ha, both in post-emergence.

Parameter	Biotype	Treatment	Slope[a]	XR_{50}[b]	XR_{90}[c]	RI_{50}	RI_{90}	R^2
Survival	S	− Malathion	−1.572	1440	4,381	1.00	1.00	0.999
		+ Malathion	−1.425	1426	6,704	0.99	1.53	0.980
	ES-R 3	− Malathion	−1.309	1586	8,785	1.10	2.01	0.981
		+ Malathion	−1.354	1408	7,092	0.98	1.62	0.982
	ES-R 2	− Malathion	−1.412	3237	15,558	2.25	3.55	0.973
		+ Malathion	−2.162	1764	4,254	1.22	0.97	0.998
	ES-R 1	− Malathion	−2.193	4721	8,653	3.28	1.98	0.983
		+ Malathion	−3.986	1288	2,657	0.89	0.61	0.997
Fresh weight (%)	S	− Malathion	1.883	649	1,887	1.00	1.00	0.994
		+ Malathion	2.350	777	2,852	1.20	1.51	0.968
	ES-R 3	− Malathion	1.803	943	3,740	1.45	1,98	0.999
		+ Malathion	1.241	743	2,903	1.14	1.54	0.992
	ES-R 2	− Malathion	2.902	965	5,969	1.49	3.16	0.986
		+ Malathion	2.525	961	2,545	1.48	1.35	0.995
	ES-R 1	− Malathion	2.445	2200	5,431	3.39	2.88	0.999
		+ Malathion	8.239	1188	1,594	1.83	0.84	0.997

XR_{50} and XR_{90} are expressed as g a.i./ha of prosulfocarb. RI, resistance index as $RI_{50} = XR_{50}(R)/XR_{50}(S)$ or as $RI_{90} = XR_{90}(R)/XR_{90}(S)$.
[a] *Slope at the XR50.*
[b] *XR_{50} represents LD_{50} for survival, and GR_{50} for fresh weight (%), herbicide concentration for 50% reduction of L. rigidum survival and fresh weight, respectively.*
[c] *XR_{90} represents LD_{90} for survival, and GR_{90} for fresh weight (%), herbicide concentration for 90% reduction of L. rigidum survival and fresh weight, respectively.*

(a CYP450s inhibitor) in the three HR populations were determined. When malathion was applied alone at 1,000 g ha^{-1}, there was no effect on survival or growth in either the S or R populations. When prosulfocarb was applied post-emergence after a pre-treatment with malathion on the S population, survival and biomass were unaffected by the insecticide (**Table 5** and **Figure 4**). RI for these two parameters, both based on LD_{50}/GR_{50} or LD_{90}/GR_{90}, ranged between 0.99 and 1.53 for the S population (**Table 5**). In the presence of malathion, ES-resistant 1 and 2 populations became more susceptible to prosulfocarb (**Table 5** and **Figure 4**); the RI_{50} for percentage of survival went down from 3.28 to 0.89 and from 2.25 to 1.22, respectively, and RI_{90}, from 1.98 to 0.61 and from 3.55 to 0.97; for percentage fresh weight reduction, RI_{50} went down from 3.39 to 1.83 and

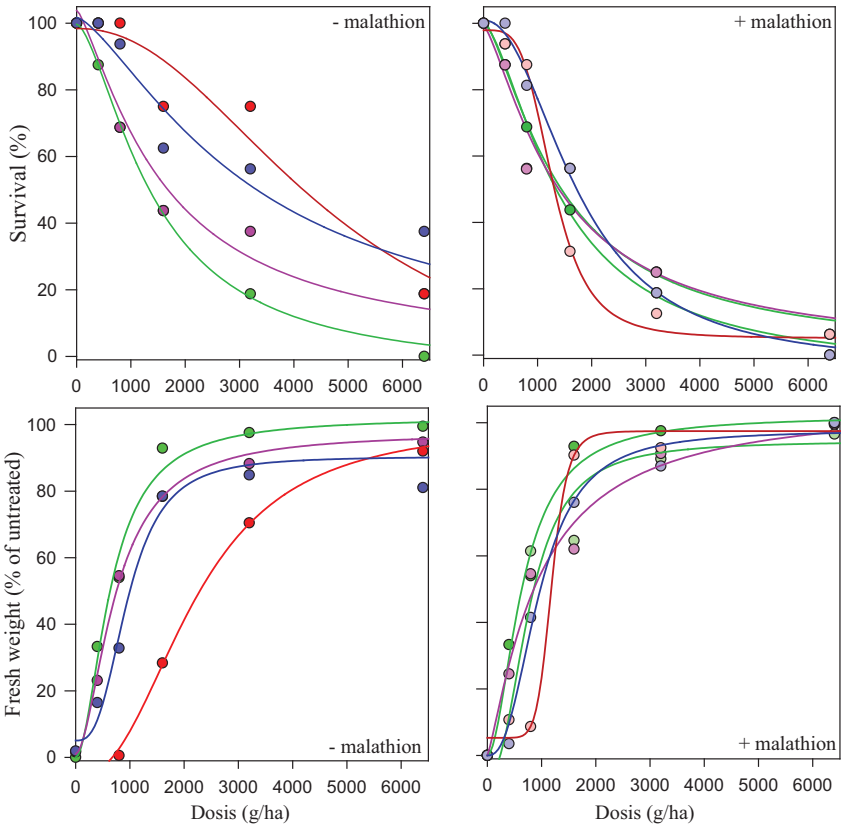

FIGURE 4 | Dose-response regression curves of prosulfocarb without malathion (– malathion, left hand graphs), or with a pre-application of malathion (right hand graphs) at a dose of 1,000 g/ha (+ malathion) in susceptible (S, green lines and dots) and resistant populations ES-resistant 3 (pink lines and dots), ES-resistant 2 (blue lines and dots) and ES-resistant 1 (red lines and dots), of *Lolium rigidum*. The *y* axis shows both the percentage of survival (upper boxes) and the effect on percentage of the mean fresh weight (lower boxes) as compared with untreated control plants. Dashed (+ malathion) and solid (– malathion) lines represent predicted values derived from the regression analysis.

from 2.88 to 0.84, respectively (**Table 5**). In contrast, malathion did not synergize prosulfocarb significantly in the ES-resistant 3 population. Thus, the survival, or effect on fresh weight (based on LD_{50}/GR_{50} or LD_{90}/GR_{90}) and RI ranged between 0.98 and 2.01, with or without the CYP450 inhibitor in the ES-resistant 3 population.

Visual inspection of treated ES-resistant 1 and 2 plants comparing both treatments (± malathion pre-treatment), revealed that survival and growth were partially reduced in the presence of the insecticide, particularly at higher prosulfocarb rates. This partial reversion of the phenotype was not observed for the ES-resistant 3 population or the S standard population (**Figure 5**). This suggested that, besides the role showed by CYP450, a secondary NTSR mechanism that did not involve these enzymes was exhibited in these plants.

DISCUSSION

Resistance to two chemical classes of ACCase-inhibiting herbicides (group 1 HRAC/WSSA), APP (diclofop) and CHD (clethodim), and to families of ALS-inhibiting herbicides (group 2), SU (mesosulfuron + iodosulfuron) and TRP (pyroxsulam + florasulam) has been confirmed in three HR *L. rigidum* populations from Spain. Furthermore, cross-resistance, or reduced susceptibility, to other SoA including the PS II inhibitor chlortoluron (group 5) and to the fatty acid elongase inhibitor prosulfocarb (group 15) was also observed in these HR populations. These are the first confirmed reports of multiple HR and cross-resistance in *L. rigidum* to these SoA in Spain and Europe. Field-evolved resistance to different pre-emergence herbicides, including prosulfocarb, has previously been confirmed in Australia (Brunton et al., 2018). Together, these findings further emphasize the plasticity of *L. rigidum* to develop resistance mechanisms to broad spectrum of herbicides.

Non-target-site resistance is a generalist resistance mechanism that develops independently from TSR, a specialist resistance mechanism that confers resistance to only one herbicide SoA, with several grasses exhibiting both classes of resistance (Comont et al., 2020). EMR is one of the best-known mechanisms underpinning NTSR and confers broad spectrum resistance to multiple herbicide SoA in weeds (Ghanizadeh and Harrington, 2017; Gaines et al., 2020).

FIGURE 5 | Visual injury of four *Lolium rigidum* populations 28 days after treatment with prosulfocarb (+ malathion, lower boxes), or without (– malathion, upper boxes) a pre-treatment with malathion (1,000 g a.i./ha). Prosulfocarb was applied at five rates (6,400, 3,200, 1,600, 800, 400, and 0 g a.i./ha) as arranged from the right to left hand side in each case. Left upper box, susceptible population (ES-S); left right, resistant population (ES-R 3); bottom left, resistant population (ES-R 2); bottom right, resistant population (ES-R 1).

Resistance to different chemical classes of ACCase-inhibiting herbicide in *L. rigidum* can result from TSR, or/and NTSR mechanisms (Takano et al., 2021). Point mutations at Trp-2027 and Asp-2078 in the ACCases of the two HR populations ESR-1 and ESR-2 were determined, with no TSR-conferring mutations found at any of the four positions tested in the HR population ES-resistant 3. Mutations at position Asp-2078 in the ACCase gene is one the most common mutation conferring TSR across a range of grass weed species. Asp-2078 is located close to the active, with mutations at this position conferring resistance to multiple chemistries acting on this enzyme (Gaines et al., 2020). Apart from Asp-2078 position, mutations in seven alternative positions in the ACCase gene are linked to TSR toward differing classes of ACCase-inhibiting herbicides in various weed species (Kaundun, 2014; Murphy and Tranel, 2019). Therefore, it is important to test for the other two ACCase positions described in *L. rigidum* (Cys2088 and Gly2096) before the final assessment of the impact of point

mutations on the resistance to ACCase-inhibiting herbicides in these HR populations can be concluded. However, based on the broad resistance to herbicides acting on multiple SoA observed in the ES-resistant 3 population, it would appear most likely that NTSR was responsible for tolerance to diclofop and clethodim in these plants. Furthermore, the level of AmGSTF1 ortholog protein (LrGSTF1) was significantly elevated in this population compared to the sensitive population which further pointed to NTSR mechanisms being activated in this *L. rigidum* population (Cummins et al., 2013). Further experiments are required to identify the specific NTSR mechanisms in these plants as previous studies have shown that reduced herbicide absorption could be one of the mechanisms that confer resistance to ACCase-inhibiting herbicide in Spanish *L. rigidum* populations (De Prado et al., 2005).

The three HR populations also showed resistance toward two chemical classes of ALS-inhibiting herbicides. To test for mutations giving rise to TSR, we found no mutation at Trp-574 position in any of the three HR populations. There are four further mutations in ALS gene of *L. rigidum* that can confer resistance to herbicides acting on this enzyme, including the common mutation at Pro-197 (Yu et al., 2008; Murphy and Tranel, 2019; Tranel et al., 2020). We could not confirm whether there is the mutation at Pro-197 using LAMP assay due to the variation of the sequences in the region surrounding Pro-197 in *L. rigidum*. As LAMP assay requires six primers to detect six distinctive regions around the sequence of interest, the Pro-197 primers produced low signal when *L. rigidum* DNA was used as template. The new design of LAMP primers to detect the mutation at Pro-197 in *L. rigidum* is required for future studies. Regardless of Pro-197 mutation, the DNA sequencing of ALS gene to identify the mutations at additional positions in the ALS is required to provide conclusive evidence of the potential for TSR contributing to resistance to ALS-inhibiting herbicides in these HR populations. However, it is also probable that there are no TSR mutations in their ALS gene. As mentioned previously, considering the broad resistance to different ALS inhibiting chemistries shown by these populations, it is unlikely the presence of mutations in position Pro-197. Moreover, in north-eastern Spain, grass control was based on chlortoluron for several decades. This scenario usually drives a selection pressure linked to EMR through enhanced CYP450 expression as being the main NTSR mechanisms (Gaines et al., 2020). Subsequent selection pressure with ALS-inhibitors would not have necessarily selected for TSR, since enhanced CYP450 activity underpinning NTSR already conferred cross-resistance to multiple herbicides in these *L. rigidum* populations.

Apart from enhanced resistance to ACCase and ALS-inhibiting herbicides, the HR populations also showed moderate levels of cross-resistance, or reduced susceptibility, to the PS II inhibitor (chlortoluron) and a thiocarbamate that inhibits fatty acid elongase (prosulfocarb). As the mutation (TSR) that confer resistance to these herbicides remain largely un-recorded in grass weeds including *L. rigidum*, it is probable that NTSR/EMR would be the resistance mechanism toward these herbicides. Dose-response experiments with prosulfocarb in the presence of the CYP450 inhibitor malathion, indicated that CYP450 could potentially contribute to these reduced susceptibilities prosulfocarb observed in these HR populations. It will now be of interest to test the effect of inhibiting CYP450 via malathion using chlortoluron, a herbicide where CYP450 have already been demonstrated as being involved in EMR in *L. rigidum* populations (Gaines et al., 2020). It is noteworthy that we observed an antagonistic effect when prosulfocarb was applied pre-emergence with malathion in the whole plant experiments (**Table 6**). This antagonism could be due to the fact that prosulfocarb is a pro-herbicide activated by CYP450 in plants (Fuerst, 1987). Therefore, the synergism (increase sensitivity in HR populations) and antagonism effects could be accounted for by the differential inhibition of diverse CYP450 by malathion. Finally, differential expression of diverse CYP450 in roots and shoots could also contribute to this phenomenon, the inhibition of enzymes responsible of herbicide activation when soil applied or enzymes responsible of prosulfocarb degradation when foliar applied.

TABLE 6 | Survival (%) in susceptible (S) and potential prosulfocarb resistant *Lolium rigidum* populations (ES-R 1 and 2) for prosulfocarb with (+ malathion) or without (− malathion) a pre-application of malathion at 1,000 g/ha, applied in pre-emergence.

Dose (g a.i. ha^{-1})			Resistant	
	Malathion	Susceptible	ES-resistant 1	ES-resistant 2
0	−	100 ± 0	100 ± 0	100 ± 0
	+	100 ± 0	100 ± 0	100 ± 0
1,200	−	13 ± 13	70 ± 12	50 ± 20
	+	25 ± 10	88 ± 13	70 ± 14
2,400	−	0 ± 0	50 ± 12	38 ± 13
	+	13 ± 7	75 ± 14	63 ± 24
4,000	−	0 ± 0	38 ± 13	13 ± 13
	+	0 ± 0	50 ± 20	25 ± 14

*Significant factors: population, malathion, and dose; among interactions only population*dose. Therefore, there are no differences between malathion treatments within each population and dose.*

Besides CYP450, previous studies have indicated that the GST enzyme superfamily can mediate EMR in *L. rigidum* toward thiocarbamate herbicides belonging to the same group (15) as prosulfocarb, through enhanced detoxification following conjugation with glutathione (GSH) (Busi et al., 2018; Dücker et al., 2019). Levels of *AmGSTF1* ortholog protein (*LrGSTF1*) were elevated in the HR populations and since this enzyme has low glutathione conjugating activity toward herbicides, its presence is linked to the increased expression of other GST (Cummins et al., 2013). As it has been reported that the tau class (U) GST (GSTU) metabolized the group 15 herbicide flufenacet in *L. rigidum* (Dücker et al., 2019), it is possible that these enzymes also contribute to EMR toward prosulfocarb and other SoA herbicides (Ghanizadeh and Harrington, 2017; Gaines et al., 2020).

The *L. rigidum* populations tested here have evolved resistance to ACCase and ALS-inhibiting herbicides and are not well-controlled by other selective herbicides. Our findings suggest that both TSR and NTSR mechanisms are present in these

L. rigidum populations which highlights the difficulties in effectively using other selective herbicide SoA to control multiple HR populations in diverse cropping systems. Altogether, the presence of ACCase and ALS HR *L. rigidum* populations in Spain, together with the confirmed presence of glyphosate-resistant (Fernandez-Moreno et al., 2017; Fernández-Moreno et al., 2017) and flufenacet-resistant populations (Dücker et al., 2019), should alert Spanish growers to the risk of multiple herbicide resistance evolving in Spanish *L. rigidum* to most available SoA on a large scale. This study confirmed the need for resistance monitoring in *L. rigidum* in Spain and the use of non-chemical control methods and integrated weed management to be adopted as preventative strategies (Cirujeda and Taberner, 2010). The precise identification of the types of resistance and the underlying molecular mechanisms that are evolving in field populations is essential to slowing, or preventing the evolution of TSR and NTSR in *L. rigidum* as well as other weed species. As such, the detection of the ortholog of *AmGSTF1* and the LAMP assay for SNP detection are promising technologies for the rapid monitoring of TSR and NTSR in *L. rigidum*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JT, JM, and AT provided the plant material and wrote the first draft. JT, JM, RE, and NO carried out the experiments. All authors contributed to the experimental design, participated in data curation and analysis, and involved in the final preparation and review of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.625138/full#supplementary-material

Supplementary Table 1 | The melting temperature of a specific product from wild type or SNP of ALS or ACCase enzyme detected by LAMP assay in this study.

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Distribution of Glyphosate-Resistance in *Echinochloa crus-galli* Across Agriculture Areas in the Iberian Peninsula

*José G. Vázquez-García[1], Antonia M. Rojano-Delgado[1], Ricardo Alcántara-de la Cruz[2], Joel Torra[3], Ignacio Dellaferrera[4,5], João Portugal[6,7] and Rafael De Prado[1]**

[1]Department of Agricultural Chemistry and Edaphology, University of Córdoba, Córdoba, Spain, [2]Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil, [3]Department d'Hortofruticultura, Botànica i Jardineria, Agrotecnio, Universitat de Lleida, Lleida, Spain, [4]Faculty of Agricultural Sciences, National University of the Litoral, Esperanza, Argentina, [5]National Scientific and Technical Research Council, Godoy Cruz, Argentina, [6]Biosciences Department, Polytechnic Institute of Beja, Beja, Portugal, [7]VALORIZA-Research Centre for Endogenous Resource Valorization, Polytechnic Institute of Portalegre, Portalegre, Portugal

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Dae-Kyun Ro, University of Calgary, Canada

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***Correspondence:**
Rafael De Prado
qe1pramr@uco.es

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The levels of resistance to glyphosate of 13 barnyard grass (*Echinochloa crus-galli*) populations harvested across different agriculture areas in the Southern Iberian Peninsula were determined in greenhouse and laboratory experiments. Shikimate accumulation fast screening separated the populations regarding resistance to glyphosate: susceptible (S) E2, E3, E4, and E6 and resistant (R) E1, E5, E7, E8, E9, E10, E11, E12, and E13. However, resistance factor (GR_{50} E1–E13/GR_{50} E6) values separated these populations into three groups: (S) E2, E3, E4, and E6, (R) E1, E5, E7, E8, and E9, and very resistant (VR) E10, E11, E12, and E13. ^{14}C-glyphosate assays performed on two S populations (E2 and E6) showed greater absorption and translocation than those found for R (E7 and E9) and VR (E10 and E12) populations. No previous population metabolized glyphosate to amino methyl phosphonic acid (AMPA) and glyoxylate, except for the E10 population that metabolized 51% to non-toxic products. The VR populations showed two times more 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity without herbicide than the rest, while the inhibition of the EPSPS activity by 50% (I_{50}) required much higher glyphosate in R and VR populations than in S populations. These results indicated that different target-site and non-target-site resistance mechanisms were implicated in the resistance to glyphosate in *E. crus-galli*. Our results conclude that resistance is independent of climate, type of crop, and geographic region and that the level of glyphosate resistance was mainly due to the selection pressure made by the herbicide on the different populations of *E. crus-galli* studied.

Keywords: barnyard grass, enhanced metabolism, glyphosate, non-target-site resistance (NTSR), resistance mechanisms, target-site resistance (TSR)

INTRODUCTION

Weeds are the main constraint in global food production and have a pivotal role in reducing quality and yield in the most important crops worldwide (Oerke, 2006). Weed control strategies have been constantly changing over recent decades through cropped areas with a tendency to monoculture without herbicide rotation, such as perennial crops, or large irrigated and horticultural crops. This scenario has provoked a decrease in herbicide efficacies due to the evolution of weed resistant biotypes. In particular, there was a quick shift in cases of weed species resistant to the single 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibiting herbicide glyphosate (group 9, HRAC and WSSA), currently the most extensively herbicide used over the world (Baylis, 2000). Since the evolution of a glyphosate resistant (GR) weed was reported for the first time (Pratley et al., 1996), 51 weed species were documented to have populations with evolved herbicide resistance over millions of hectares of the best crop producing areas around the globe (Heap, 2020). Glyphosate has been widely used in GR crops in many American countries, while this herbicide is used especially in the European Mediterranean in perennial crops, corn, and rice in direct sowing and large horticultural crops, among others (Antier et al., 2020). It is well-known that glyphosate is a non-selective herbicide that it is absorbed through leaves. The enzyme EPSPS (EC 2.5.1.19) is the target-site of glyphosate in plants. This enzyme catalyzes, in the shikimic acid pathway, the conversion of phosphoenolpyruvate and shikimate-3-phosphate into inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate. Its inhibition prevents the biosynthesis of phenylalanine, tyrosine, and tryptophan, aromatic amino acids (Franz et al., 1997). The resistance mechanisms are broadly divided into non-target-site resistance (NTSR) and target-site resistance (TSR) (Gaines et al., 2020). TSR implies conformational changes in the target-proteins of herbicides that result from deletion or amino acid substitution, but also gene overexpression or amplification that increases target protein abundances (Gaines et al., 2020). NTSR covers those mechanisms not related to the enzymes targeted by herbicides. Often, NTSR mechanisms act reducing to a sublethal dose the herbicide that reaches a target protein and may involve reduced absorption/translocation of the herbicide, vacuolar sequestration, or enhanced metabolism (metabolic herbicide resistance) (Ghanizadeh and Harrington, 2017).

The Iberian Peninsula, with more than 5,000,000 ha, followed by Italy (2,500,000 ha), was the most important member state of the EU-28 Mediterranean Region in terms of perennial, corn, and rice crops in direct sowing and large horticultural crops in 2017 (Antier et al., 2020). The common climate, absence of crop rotation, and few herbicides being widely used resulted in their fields having similar glyphosate resistant weeds. Currently, *Conyza bonariensis*, *Conyza canadensis*, *Conyza sumatrensis*, *Hordeum murinum, Lolium multiflorum*, *Lolium perenne*, *Lolium rigidum*, and *Sorghum halepense* have evolved resistance to glyphosate in Iberian Peninsula (Heap, 2020). Nevertheless, since 2018, farmers have been complaining about the appearance of a new glyphosate resistant grass species, identified as *Echinocloa crus-galli*.

Echinochloa crus-galli (L.) P. Beauv is an annual C4 grass weed reported as a hexaploid species, whose karyotype is $2n = 6x = 54$ chromosomes (Ye et al., 2020). The plant has dull green leaves often with conspicuous anthocyanin pigmentation, glabrous compressed sheaths, with no ligules and auricles; they form a clump with prostrate tillers reaching up to 150 cm in height and reproduces by caryopses disposed in erected panicles (Maun and Barrett, 1986; Damalas et al., 2008). Fertilization occurs mainly by self-pollination; however, a certain degree of crossbreeding can occur, facilitated by wind. High levels of homozygosity within populations result from self-fertilization together with a relatively low degree of heterozygosity in polymorphic loci (Maun and Barrett, 1986). It has a high tillering capacity, being also a very prolific species (Owen et al., 2020); these characteristics, added to the fact that seeds can easily disperse, are dormant, and it can flower under a wide photoperiod range, make it a very successful weed (Maun and Barrett, 1986). This species has biological and ecological similarities with rice and for this reason is one of the main rice weeds all over the world (Tian et al., 2020), but in the Iberian Peninsula it also acts as weed in soybean, maize, and other crops (Dorado et al., 2009). This is a particular concern because it is among the top 15 weed species with herbicide resistance worldwide (Yang et al., 2017) with cases reported in 23 countries, principally in rice but also in other crops, such as corn, orchards, and perennial crops. Among the herbicidal modes of action to which *Eleusine indica* has been reported as being resistant are the inhibitors of the acetolactate synthase, acetyl-CoA carboxylase, 1-deoxy-D-xyulose 5-phosphate synthase, EPSPS, photosystem II, cellulose, lipids, microtubules, a very long chain fatty acid, as well as synthetic auxins (Heap, 2020).

This study determined whether *E. crus-galli* populations, infesting several perennial and annual crops in the Iberian Peninsula, are resistant to glyphosate, as well as the resistance mechanisms present, particularly NTSR mechanisms (absorption, translocation, and metabolism). EPSPS enzyme activity data were used to infer putative TSR mechanisms present in the studied populations.

MATERIALS AND METHODS

Plant Material

Mature seeds of *E. crus-galli* were collected between 2018 and 2019 in perennial crop fields (olive, citrus, vineyards, and pomegranates-tree) and annual crops (rice and corn) from the south of the Iberian Peninsula (**Table 1**), where farmers reported control failures of this species with glyphosate after more than 10 years of application. Thirteen populations were collected and taxonomically characterized, and we obtained historical records of field application only for some populations due to a lack of good record keeping in other cases. The seeds of each population were harvested from at least 25 adult plants in georeferenced 50 m^2 areas (**Table 1**). Seeds were cleaned and stored at 4°C for further testing. Germination of the different populations was very irregular and was between 40 and 80%.

The climate in central Andalusia (Southern Spain) and Alentejo (Center of Portugal) typically has long, hot, and arid

TABLE 1 | Distribution of *Echinochloa crus-galli* across agriculture areas in the Southern Iberian Peninsula and its main management characteristics with glyphosate.

Pop.	Crop	Country	GPS coordinates	Year application[a]/dose[b]	Year harvested
E1	Olive grove	Spain	37°40'32.5"N 4°14'23.0"W	10/720	2018
E2	Citrus orchard	Spain	37°42'06.6"N 5°18'48.7"W	Organic	2019
E3	Olive grove	Spain	37°31'05.7"N 4°50'30.9"W	5/540	2018
E4	Olive grove	Spain	37°42'30.3"N 4°30'45.3"W	3/540	2018
E5	Orchard	Spain	37°38'06.9"N 4°21'54.1"W	15/540–720	2019
E6	Runnel (non crop)	Portugal	38°01'12.4"N 7°46'08.0"W	No	2019
E7	Citrus orchard	Spain	37°45'24.2"N 5°15'56.9"W	12/1080	2019
E8	Citrus orchard	Spain	37°41'57.7"N 5°18'28.7"W	10/720	2019
E9	Rice	Spain	36°22'16.0"N 5°52'40.6"W	12/1080	2019
E10	Pomegranates-tree	Portugal	38°06'02.4"N 7°49' 21.9"W	15/1080	2019
E11	Corn	Spain	36°19'32.1"N 5°47'34.5"W	12/720-1080	2019
E12	Corn	Portugal	37°54'54.5"N 8°21'47.8"W	15/1080	2019
E13	Vineyard	Portugal	37°54'13.2"N 7°58'23.8"W	12/720-1080	2019

[a]*Usually farmers applied two times year^{-1} in perennial crops, in the last time (5 years) in autumn, herbicides such as flazasulfuron [acetolactate synthase (ALS) inhibitor] and oxyfluorfen [protoporphyrinogen oxidase (PPO) inhibitor] plus glyphosate are applied. In spring, commonly MCPA plus glyphosate are applied. On the other hand, in annual crops such as rice and corn, the glyphosate is applied only one time cicle^{-1} in pre-sowing.*
[b]*g ae ha^{-1}.*

summers and winters that are short, cold, and partly cloudy. Throughout the year, the temperature generally varies from 6 to 35°C and rarely drops below 2°C or rises above 45°C. All fields where seeds were collected were irrigated with river or swamp water that ranges between 1,500 and 6,000 L ha^{-1}. The types of soils were highly variable between sandy and clay.

Fifteen-cm-diameter petri dishes were conditioned with two layers of moistened (5 ml distilled water) filter paper to germinate the seeds of the *E. crus-galli* populations. Petri dishes were kept in a germination chamber calibrated at 28/18°C (day/night), 16-h photoperiod, 850 μmol m^{-2} s^{-1} light intensity, and 80% relative humidity. Once germinated, seedlings were transplanted individually in 250 ml punnet pots (peat/sand, 2:1 v/v) and taken to a greenhouse maintaining the same temperature and photoperiod regime as in the germination chamber (Fernández-Moreno et al., 2017a).

Shikimate Accumulation Fast Screening

Five samples (50 mg of 4 mm diameter leaf discs) of each *E. crus-gaalli* population were taken from a pool of young and fully expanded leaves from at least 10 plants (Vázquez-García et al., 2020a). Leaf discs of each sample were saved in 2 ml tubes containing 1 ml of different glyphosate concentrations (0 and 1000 μM) prepared in 1 mM ammonium dihydrogenphosphate (pH 4.4). Sample tubes were incubated at 25°C and light intensity of 850 μmol m^{-2} s^{-1}. Shikimic acid was extracted following the methodology of Shaner et al. (2005). Accumulation was estimated from the difference between the shikimic acid concentration in treated and untreated plants based on a calibration curve with known concentrations of standard shikimic acid (Sigma-Aldrich Co., Saint Louis, MO, United States). Two technical replicates were analyzed from each sample and the results were expressed in μg g^{-1} fresh weight.

Dose-Response Assays

Plants at the three to four leaf stages of the *E. crus-galli* populations were treated with nine glyphosate (Roundup Energy, 450 g ae L^{-1} as isopropylamine salt) doses ranging from 0 to 3,000 g ae ha^{-1}. Herbicide applications were done in a herbicide treatment cabinet with output volume of 200 L ha^{-1} at a pressure of 250 kPa. Moving-boom of the cabinet has a Teejet 8002-EVS nozzle positioned 50 cm above the plant canopy. Sets of 10 plants of each population were treated for each dose of herbicide, and the experiments were repeated twice. Herbicide response (weight reduction and mortality) were determined 21 days after the treatments (DAT) and transformed in percentage with respect to the controls (Vazquez-Garcia et al., 2020b).

^{14}C-Glyphosate Uptake and Translocation

The second or third leaf of eight plants (five and three for quantitative and qualitative analyzes, respectively) of the E2, E6, E7, E9, E10, and E12 populations was covered with aluminum envelopes. Plants were sprayed with 360 g ae ha^{-1} of formulated glyphosate (cold treatment) and 30 min later, once herbicide solution dried, the aluminum was removed. After, 1-μl drop of ^{14}C-glyphosate (glycine-2-^{14}C, 95% radiochemical purity, 273.8 MBq mmol^{-1}specific activity, Institute of Isotopes Co., Ltd., Budapest, Hungary) + formulated glyphosate (hot treatment) per plant was deposited on the adaxial surface of these leaves using a micro syringe (Hamilton PB6000 Dispenser). The hot solution had 100,000 dpm μl^{-1} specific activity and 360 g ae ha^{-1}. Four DAT, the non-uptake ^{14}C-glyphosate was washed three times with water: acetone (1:1 v/v; 1 ml each time). Wash solutions were recovered in ml scintillation vials and 2 ml of scintillation cocktail was added.

Plants were removed from the punnet pots and impurities in the roots were carefully washed with distilled water. Quantitative analysis plants were sectioned into treated leaf, rest of the aerial part of the plant, and roots. Plant sections were saved in filter paper cones, dried at 60°C during 4 days and burned individually in an automatic oxidizer (Packard Tri Carb 307, Packard Instruments, Meriden, United States) during 3 min. The $^{14}CO_2$ released during combustion was captured in 18 ml of radioactive dioxide absorber solution (Carbosorb-E®,

Perkin-Elmer) and liquid scintillation cocktail (Permafluor®, Perkin-Elmer; 1:1, v/v). Radioactivity of wash solutions and combustion was quantified by liquid scintillation spectrometry (10 min). Experiments had a randomized design and the absorption and translocation percentages were calculated according to Alcántara-de la Cruz et al. (2021).

The three plants of each population reserved for the qualitative analysis of ^{14}C-glyphosate translocation were fixed on filter paper sheets (12.5 cm × 25 cm), pressed and dried at room temperature for 1 week. The dried plants were then exposed to a phosphor storage screen for 13 h in the dark. Radioactivity distribution within plants was scanned in a storage phosphor system (Cyclone Plus, Perkin-Elmer).

Glyphosate Metabolism

Ten plants at the four-leaf stage of the E2, E6, E7, E9, E10, and E12 populations were sprayed with glyphosate at 360 g ae ha^{-1}. Other groups of plants (the same number of plants) were sprayed only with water to be used as control. Four DAT, whole plants were removed from the punnet pots, carefully washed with distilled water, packed in aluminum foil envelopes, and immediately frozen in liquid N_2. The samples were stored at 40°C until processing for analysis. The extraction of amino methyl phosphonic acid (AMPA), formaldehyde, glyphosate, glyoxylate, and sarcosine as well as its quantification by reversed polarity capillary electrophoresis were performed according to Rojano-Delgado et al. (2010). The concentrations of each compound were determined using calibration curves with known concentrations of standard compounds (Sigma-Aldrich, Madrid, Spain). Data were expressed as percentages of the sum of glyphosate plus metabolites recovered.

EPSPS Enzyme Activity Assays

The EPSPS activity was assayed in the E6, E7, E9, E10, and E12 populations. Leaf tissue samples were taken from four leaf stage plants up to complete 5 g per population. Samples were stored at 40°C until analyses, when they were macerated in a mortar until obtaining fine powder. The extraction of the target enzyme of the glyphosate, as well as the determination of the total soluble protein (TPS, basal activity without glyphosate) and the EPSPS inhibition rate by adding increased concentrations of glyphosate (0, 0.1, 1, 10, 100, and 1000 μM) were performed following the detailed methodology by Dayan et al. (2015). For each glyphosate concentration, three technical replicates of each population were assayed. Experiment was repeated twice and the results were given as a percentage relative to the control (0 μM glyphosate) of the amount (μmol) of inorganic phosphate (Pi) released per μg of TSP min^{-1} (μmol Pi μg^{-1} TSP min^{-1}).

Statistical Analyses

The three-parameter regression function, $y = d/\{1+\exp.[b(\log x - \log e)]\}$, was used to estimate the weight reduction, plant mortality, and EPSPS inhibition at a rate of 50% (GR_{50}, LD_{50}, and I_{50}, respectively), by fitting their respective percentage data in the "drc" package of the R software environment (Keshtkar et al., 2021). The function parameters represent: "*b*" is the relative slope of the curve, "*d*" is the upper limit of "*y*," "*e*" is the herbicide rate that reduces "*y*" by 50%, and "*y*" is the dry weight, plant survival, or EPSPS inhibition of a given population. Resistance levels (RF) were calculated for each variant of "*y*" as the ratio between the "*e*" of the resistant populations to the "*e*" of the representative susceptible population.

For the rest of the data, the normal error distribution and the homogeneity of the variance were verified for each set. Then ANOVAs were performed and when the value of p was <0.05, the means were separated by the Tukey's test.

RESULTS

Shikimate Accumulation Fast Screening

The accumulation of shikimic acid differed between *E. crus-galli* populations. The populations E2, E3, E4, and E6 accumulated high rates of shikimic acid. The highest accumulation (29.3 μg shikimic g^{-1}) was recorded at 1,000 μM glyphosate in the E6 population. Regarding populations resistant to glyphosate, we observed two groups; the first was formed by populations E1, E5, E7, E8, and E9, which accumulated low rates of shikimic acid that varied between 1.4 and 5.4 μg g^{-1} fresh weight. The second group was made up of populations E10, E11, E12, and E13 that accumulated very low rates of shikimate, ranging from 1.1 to 1.3 μg shikimic acid g^{-1} fresh weight (**Figure 1**).

Dose Response Assays

The 13 *E. crus-galli* populations were grouped in: glyphosate-susceptible (S), −resistant (R), and -very resistant (VR), considering their GR_{50}. The group of S populations (E2, E3, E4, and E6) had RF values less than 4 and the LD_{50} values were also very low and less than the field label dose (1.08 kg ae ha^{-1}). However, the nine resistant populations survived the field doses and their LD_{50} ranged from 1532 (E1) to 2892 (E10) g ae ha^{-1}. The GR_{50} values separated the resistance level into two groups; first group formed by R populations E1, E5, E7, E8, and E9 with RF values between 6.9 and 9.4 and a second group of VR populations E10, E11, E12, and E13 with RF values between 11 (E11) and 21.7 (E10) (**Table 2**; **Figure 2**).

^{14}C-Glyphosate Uptake, Translocation, and Visualization

The ^{14}C-glyphosate recovered in two S (E2 and E6), two R (E7 and E9), and two VR (E10 and E12) populations ~90–96% after 4 DAT. The uptake rate of ^{14}C-glyphosate was higher in the S populations E2 and E6 compared with the resistant populations. In addition, the S populations moved more ^{14}C-herbicide from the treated leaf to the rest of the shoots (rest of the aerial part of the plant plus root system) was shown in compared with the R and VR populations (**Figure 3**). ^{14}C-glyphosate visualization (red color) confirmed previous results (**Figure 4**).

Glyphosate Metabolism

Metabolism of glyphosate was different between *E. crus-galli* populations at 96 HAT (**Figure 5**). Specifically, the accumulation

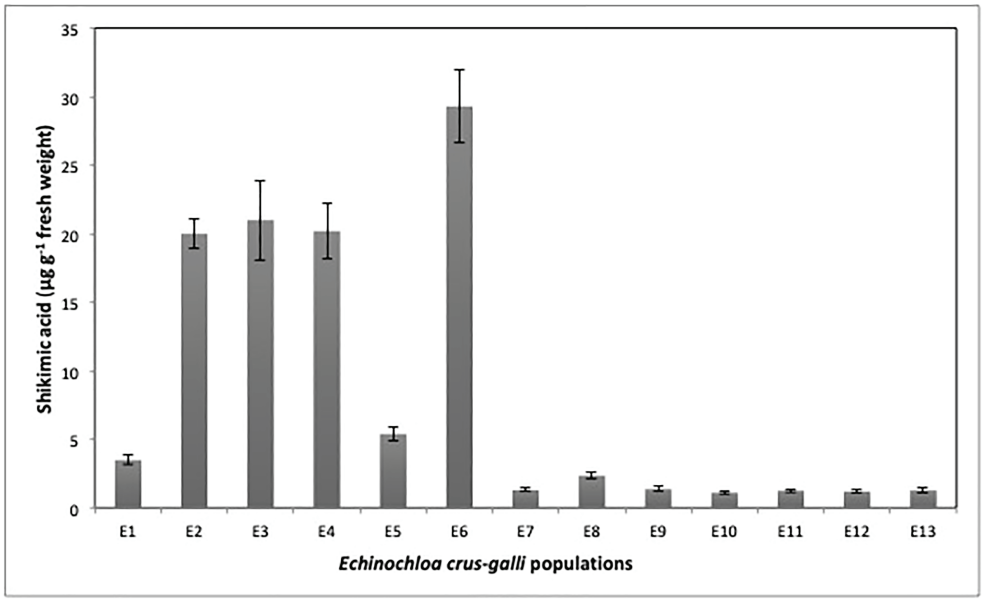

FIGURE 1 | Shikimate accumulation values in 13 *Echinochloa crus-galli* populations treated with glyphosate at 1000 µM.

TABLE 2 | Parameters[a] of the equations used to calculate the glyphosate rates (g ae ha^{-1}) required for a 50% reduction in dry weight (GR_{50}) or survival plants (LD_{50}) of 13 *Echinochloa crus-galli* populations.

Pop.	b	d	GR_{50}[b]	RF[c]	b	d	LD_{50}[b]	RF[c]
E1	1.4	91.7	317.6 ± 53.2	7.9	21.4	100.0	1532.9 ± 41.5	12.3
E2	1.6	100.7	50.9 ± 5.8	1.3	14.9	100.5	157.6 ± 11.2	1.3
E3	1.7	104.3	71.0 ± 7.2	1.8	39.3	100.0	363.8 ± 32.0	2.9
E4	1.9	99.1	69.7 ± 7.4	1.7	46.3	100.0	296.0 ± 3.6	2.4
E5	0.9	97.9	166.3 ± 32.8	6.7	36.7	100.0	1528.6 ± 66.2	12.2
E6	3.1	100.1	40.3 ± 3.0	-----	11.8	100.1	125.0 ± 4.6	----
E7	1.4	99.7	379.1 ± 47.3	9.4	26.3	100.0	2447.3 ± 10.4	19.6
E8	1.7	99.7	293.3 ± 33.1	7.3	28.9	100.0	2000.0 ± 36.5	16.0
E9	1.1	98.3	328.6 ± 55.8	8.2	43.2	100.0	2465.0 ± 20.5	19.7
E10	3.6	94.5	873.2 ± 43.6	21.7	30.2	100.0	2893.0 ± 11.4	23.1
E11	0.9	100.2	444.8 ± 8.5	11.0	74.4	100.0	2432.5 ± 12.0	19.5
E12	1.3	97.2	581.2 ± 7.3	14.4	22.9	100.0	2098.2 ± 33.7	16.8
E13	1.4	100.6	525.2 ± 11.5	13.0	22.9	99.9	2098.2 ± 36.9	16.8

[a]*$y = d/\{1+\exp[b(\log x - \log e)]\}$, where b is the relative slope of the curve, d is the upper limit of "y," e is the herbicide rate that reduces "y" by 50% and "y" is the dry weight (GR_{50}) or plant survival (LD_{50}) of a given population.*

[b]*Mean ± SEM.*

[c]*RF = Resistance factor (R/S–E6) calculated using the GR_{50} or LD_{50} values.*

of glyphosate in the E2, E6, E7, E9, and E12 populations was double that of the E10 population. The metabolism of glyphosate to AMPA and glyoxylate was 51%, while the rest of the populations studied remained unchanged and close to 90% (**Figure 5**). At least in part, metabolism had a crucial function in the response to glyphosate of the E10 population, from the VR group.

Activity of the EPSPS

The basal activity of the EPSPS differed between the six *E. crus-galli* populations studied. The populations S (E2 and E6) and R (E7 and E9) had a similar EPSPS concentrations (2.95–3.0 µmol µg^{-1} TSP min^{-1}), while the VR E10 and E12 populations had twice the target enzyme of glyphosate (6.0 µmol µg^{-1} TSP min^{-1}) (**Figure 6A**). Inhibition of the EPSPS by glyphosate in plants from the S, R, and VR populations was achieved as herbicide concentrations increased. The R populations required between 16 and 25 times more herbicide to inhibit EPSPS by 50% in relation to the most susceptible population (E6, 0.7 µM glyphosate), while in the VR, such inhibition required between 46 and 55 µM herbicide (**Table 3** and **Figure 6B**).

DISCUSSION

Andalusia and Alentejo are the biggest regions in absolute terms of irrigated area with 1,295,918 ha, 29.35% of the

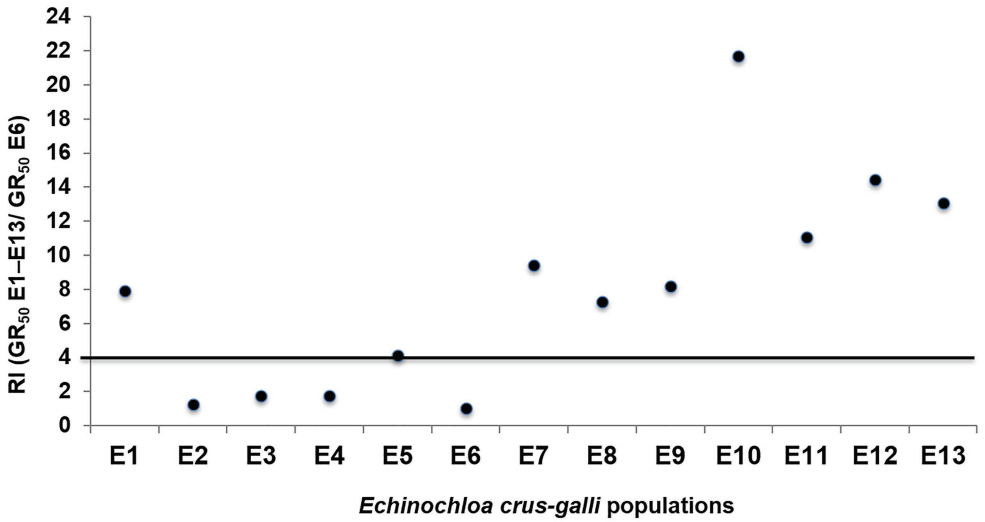

FIGURE 2 | Representation of the resistance factor (RF; GR_{50} E1–E13/GR_{50} E6) values of different *Echinochloa crus-galli* populations. Populations above the line were considered glyphosate-resistant.

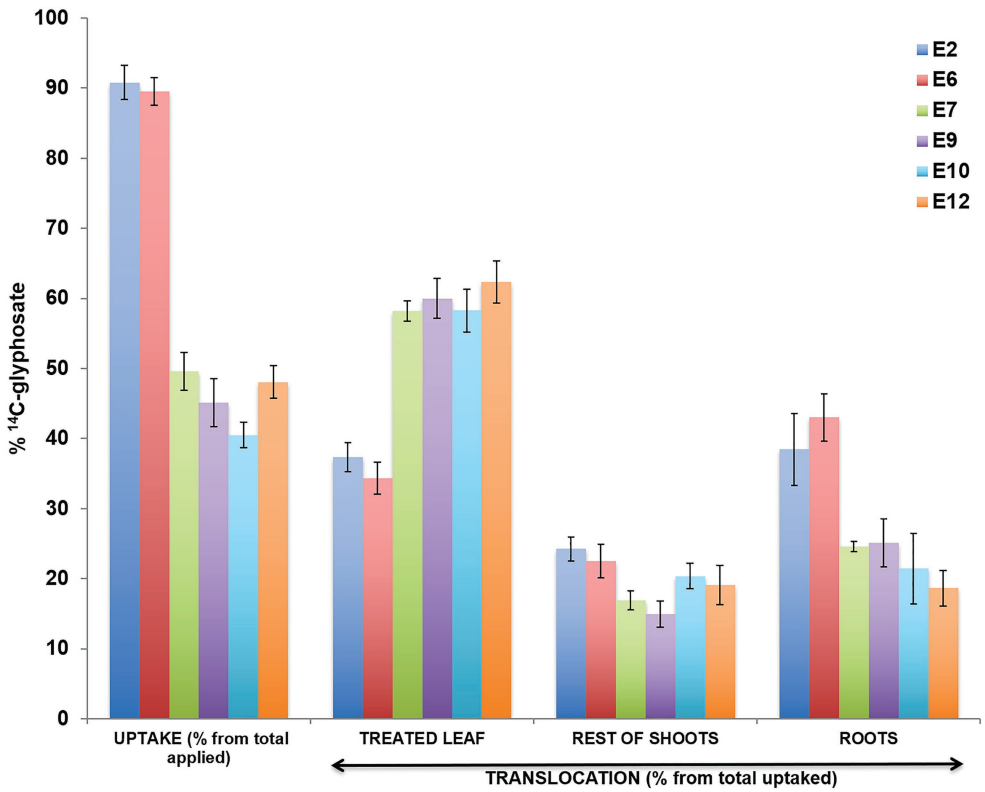

FIGURE 3 | Absorption and translocation of ^{14}C-glyphosate (%) at 96 h after treatment in different *Echinochloa crus-galli* populations, glyphosate-resistant (R; E7 and E9), -very resistant (VR; E10 and E12), and -susceptible (S; E2 and E6).

total irrigated Spanish and Portuguese area. The dominant presence of localized irrigation stands out, which has been progressively increasing and represents 75% of the total main irrigation systems in these regions. The crops with the largest irrigated area are olive groves, citrus-trees, rice (flooding irrigation), wheat, and corn under direct sowing, as well as

FIGURE 4 | Visualization of ^{14}C-glyphosate in resistant (R; E7 and E9), -very resistant (VR; E10 and E12), and -susceptible (S; E2 and E6) *Echinochloa crus-galli* populations 96 h after an application to the treated leaf.

orchards and lately, for the last 20 years, new almond-tree plantations in an intensive regime. The use of glyphosate for many years under the row in perennial crops and also in fallow fields imposed massive selection pressure on the treated weeds, leading to the emergence of resistance, mainly in Mediterranean Europe (González-Torralva et al., 2012, 2014; Fernández-Moreno et al., 2017a,b; Amaro-Blanco et al., 2018; Vázquez-García et al., 2020a,b).

Determining Resistance

Echinochloa crus-galli, a troublesome weed in rice, corn, and other perennial crops, is often controlled exclusively by chemical tools (Alarcón-Reverte et al., 2015; Nguyen et al., 2016; Fang et al., 2019; Vidotto et al., 2020). This work assessed the effect of repeated use of glyphosate in 13 populations of *E. crus-galli*. Using the accumulation rate of shikimic acid due to the EPSPS activity inhibition, it was observed that S populations significantly increased their shikimic level with respect to the putative resistant populations. This rapid screening allowed the separation of different levels of glyphosate susceptibility: S to glyphosate E2, E3, E4, and E6 and R- E1, E5, E7, E8, and E9, and VR- E10, E11, E12, and E13. The low values of GR_{50} and LD_{50}, as those observed in S populations, are due to the fast and greater inhibition of the EPSPS, which results in a high accumulation of shikimate (Shaner et al., 2005). Inversely, low susceptibility to glyphosate and consequently little accumulation of shikimic acid, as observed in the R and VR *E. crus-galli* populations were consistent with the presence of one or more herbicide resistance mechanism, as found in different grass weed species (de Carvalho et al., 2012; Alarcón-Reverte et al., 2015; Vázquez-García et al., 2020b). This research also concluded that RF based in GR_{50} values separated these 13 populations

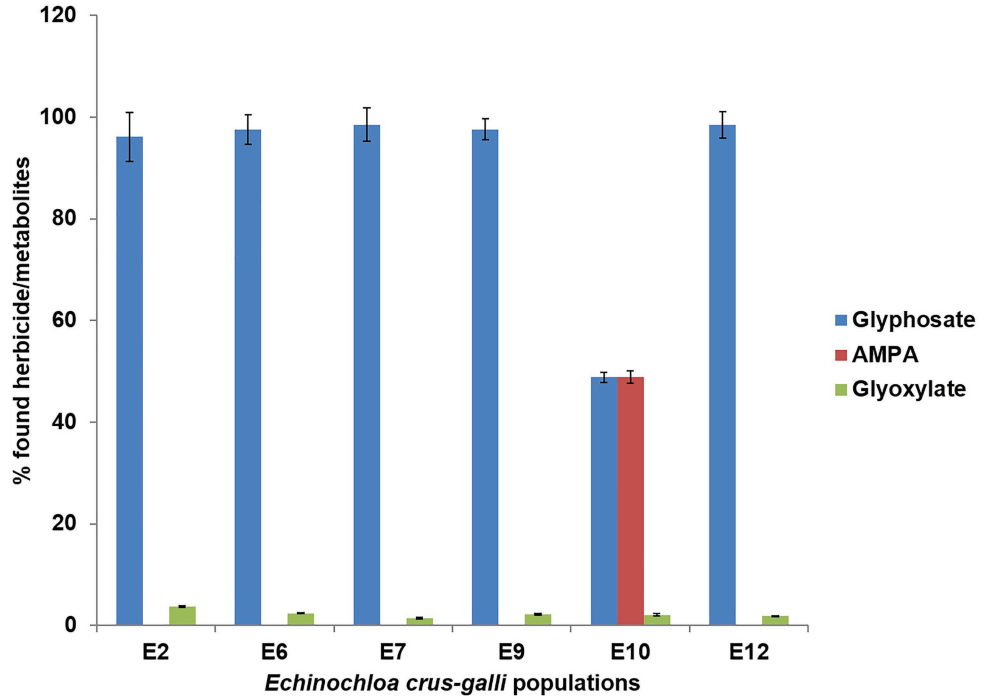

FIGURE 5 | Glyphosate metabolism in glyphosate-resistant (R; E7 and E9), -very resistant (VR; E10 and E12), and -susceptible (S; E2 and E6) *Echinochloa crus-galli* plants 96 h after application at 360 g ae ha^{-1}.

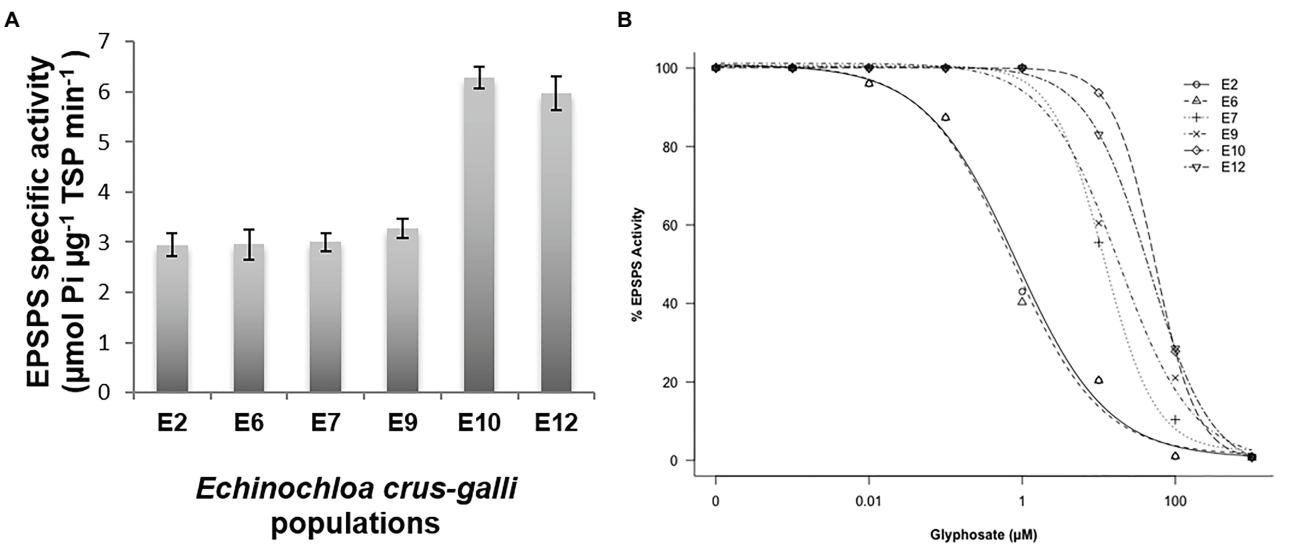

FIGURE 6 | 5-enolpyruvylshikimate-3-phosphate synthase activity in glyphosate-susceptible (S; E2 and E6), -resistant (R; E7 and E9), and -very resistant (VR; E10 and E12) *Echinochloa crus-galli* populations. **(A)** Mean of Basal EPSPS activity for glyphosate-susceptible and -resistant populations (n = 6). **(B)** EPSPS enzyme activity expressed as the percentage of the untreated control in leaf extracts of plants.

in three groups, S, R, and VR (**Figure 2**). All resistant populations had values greater than 4, a requirement to be considered resistant (Heap, 2020; Vázquez-García et al., 2020a). In addition, the LD_{50} is widely employed to determine the herbicide rate need to kill the individuals of a weed population at 50%. Glyphosate label field dose recommended in Spain and

TABLE 3 | Parameters of the equations and glyphosate concentrations (μM) required for a 50% reduction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity in different *Echinochloa crus-galli* populations.

Efficacy level	Population	d	b	R^2	I_{50} (μM)	RF
Susceptible	E2	100.8	0.73	0.999	0.8	1.1
Susceptible	E6	100.9	0.75	0.999	0.7	--
Resistant	E7	100.7	1.30	0.999	11.7	15.8
Resistant	E9	101.2	0.90	0.999	18.1	24.5
Very Resistant	E10	100.0	1.58	0.999	54.8	74.0
Very Resistant	E12	100.3	1.107	0.999	45.6	61.6

RF, resistance factor ($I_{50}R/I_{50}S$).

Portugal is 1,080 g ae ha^{-1}, which efficiently controlled the S populations E2, E3, E4, and E6, but not R populations E1, E5, E7, E8, and E9 or VR populations E10, E11, E12, and E13 of *E. crus-galli* (**Table 2**). This research revealed different levels of resistance to glyphosate in *E. crus-galli* collected in different crops of two large agricultural areas in Southern Spain and Central Portugal, where there is a variety of soils and climatic conditions. Weeds from different locations frequently show a differential response to herbicide, since each one has a unique genetic and ecological background, which is governed by climatic and edaphic conditions, type of crop where the weed developed, as well as cultural management crop tasks and the history of herbicide selection, among other agroecological factors (Shaner and Beckie, 2014; Jussaume and Ervin, 2016). In addition, it should also be considered that in each country, the glyphosate-based formulations, dose, time, and number of applications a year may vary, as well as the application technology used in each farm (Neve et al., 2014; Owen, 2016). Conversely, conditions of high temperature and relative humidity can contribute to improve the absorption and translocation of glyphosate, and effectiveness in monocots (Hatterman-Valenti et al., 2011; Nguyen et al., 2016; Fernández-Moreno et al., 2017a), which could help us understand the differences between *E. crus-galli* populations.

Exploring the Mechanism Involved

The study of NTSR mechanisms was developed on two S-glyphosate (E2 and E6), two R- (E7 and E9), and two VR- (E10 and E12) populations. Epicuticular wax coating acts as an obstructive barrier against various herbicides. Some resistant and glyphosate-tolerant weeds have exhibited a non-uniform three-dimensional cover with a higher quantity of epicuticular waxes relative to their susceptible counterparts (Cruz-Hipolito et al., 2009, 2011). The E7, E9, E10, and E12 populations presented reduced absorption of ^{14}C-glyphosate. However, this parameter is little studied and only in a few cases, such as Italian ryegrass (*Lolium multiflorum*), Johnsongrass (*Shorgum halepense*), and sourgrass (*Digitaria insularis*), has it been found to contribute to the lower susceptibility to glyphosate (Michitte et al., 2007; de Carvalho et al., 2012; Vila-Aiub et al., 2012). Differences in translocation occurred because the ^{14}C glyphosate had moved nowhere once inside the leaf in R plants, whereas in S plants, glyphosate was uptake and translocated from the point of application to the rest of the shoots and roots in large quantities. Both absorption and impaired movement of glyphosate contributed to the resistance in the R and VR *E. cruss-galli* populations. It has been demonstrated in grass weeds that the main NTSR mechanisms involved in their resistance to glyphosate were those two (Vila-Aiub et al., 2012; Bracamonte et al., 2017; Gherekhloo et al., 2017).

Most plants do not have a high ability to metabolize glyphosate to non-toxic forms, favoring the death of plants. Some *Fabaceae* plants may be able to partially metabolize part of the absorbed glyphosate through glyphosate oxidoreductase (GOX), which cleaves the CN glyphosate bond forming amino methyl phosphonic acid (AMPA) and glyoxylate and, to a lesser extent, through a CP lyase, forming sarcosine and inorganic phosphate (Rojano-Delgado et al., 2010, 2012; Duke, 2011; Finley and Duke, 2020). Only four cases, among a wide range of studies on weeds resistant to glyphosate, reported metabolism as a resistance mechanism, showing evidence of glyphosate metabolites, such as AMPA or sarcosine (de Carvalho et al., 2012; González-Torralva et al., 2012; Pan et al., 2019). Among the six *E. crus-galli* populations studied, only the most resistant population E10 from the VR group was able to metabolize glyphosate (51%) to non-toxic metabolites (**Figure 5**). Aldo-keto reductase, a metabolic enzyme of plants, was found to be responsible for metabolizing glyphosate in glyphosate-resistant *Echinochloa colona* (Pan et al., 2019); however, molecular studies are necessary to establish or rule out the contribution of this enzyme in the glyphosate metabolism in the E10 *E. crus-galli* population.

Over the last two decades, research on the TSR mechanisms involved in glyphosate resistance have been carried out in a lot of monocot and dicotyledonous (Sammons and Gaines, 2014; Heap, 2020). Currently, two mechanisms within the target-site have been considered responsible for the resistance of weeds to glyphosate: (a) alteration/mutation at the encoding *EPSPS* gene that limit the interaction of glyphosate with the target enzyme and (b) overexpression/amplification of the target gene (Gaines et al., 2020). Differences between *E. crus-galli populations* in EPSPS enzyme activity were found with and without different glyphosate rates. Thus, R (E7 and E9) and VR (E10 and E12) populations had high I_{50} values (concentration of herbicide necessary to reduce EPSPS enzyme activity to 50%) with respect to the two glyphosate-susceptible E2 and E6 populations (**Table 3** and **Figure 6B**). These results suggested that E7, E9, E10, and E12 populations were candidates that possess one or more effective mutation/s altering the coupling of the herbicide to the target enzyme (Salas et al., 2015;

Fernández-Moreno et al., 2017a; Bracamonte et al., 2018; Morran et al., 2018). Additionally, the high glyphosate resistance values of VR populations E10 and E12 could be related to possible EPSPS overexpression, as suggested by a 2-fold increase in their EPSPS basal activity compared to E7 and E9 R populations. Differences in the EPSPS basal activity have already been documented in some grass weeds due to an overs-amplification of the *EPSPS* gene or even to an enhanced basal specific EPSPS activity in the absence of such amplification (Gaines et al., 2010; Alarcón-Reverte et al., 2015; Bracamonte et al., 2016). Further experiments are currently underway to unravel the TSR mechanisms present in these resistant *E. crus-galli* populations.

The close relative *E. colona* is also able to evolve different TSR and NTSR mechanisms to glyphosate, i.e., reduced translocation, point mutations, and enhanced metabolism. *Echinoclhopa colona* individuals with different and concerted TSR mechanisms were identified coexisting within different populations collected in the California Valley (Alarcón-Reverte et al., 2015). For example, some populations from Australia or United States exhibited mutations and others, reduced translocation (Nguyen et al., 2016; Nandula et al., 2018). Additionally, glyphosate metabolism has already been described in one *E. colona* population (Pan et al., 2019), which afterward also was shown to possess a Pro106Thr mutation (McElroy and Hall, 2020). Since each resistance mechanism usually confers different resistance levels, i.e., low to moderate resistance levels are associated with point mutations compared to other mechanisms (Sammons and Gaines, 2014), the evolution of one or more mechanisms within different populations should be associated mostly with differential selection pressures posed by glyphosate, among other factors. This seems to be the case for the *E. crus-galli* populations studied in this research. Two groups of populations were defined here according to the resistance levels: R and VR. Interestingly, previously, R populations survived 10–12 glyphosate applications at 720 or 1,080 g/ha, while VR ones, 12–15 applications almost always at 1,080 g/ha, according to historical herbicide records. Therefore, selection pressure was stronger with higher doses over more years in VR compared to R populations. Accordingly, reduced uptake and transport was detected in both groups, while metabolism was only detected in the most resistant VR population. Though TSR mechanisms were not investigated, EPSPS activity results suggested that mutations may be present in both R and VR populations, while overexpression might also be present in VR populations (E10 and E12), as pointed out by their ~2-fold increase in EPSPS basal activity. Future research is underway to underpin the TSR mechanisms that have evolved in these populations, which would confirm these hypotheses.

Combinations of multiple TSR and/or NTSR mechanisms in a single individual plant can also arise through outcrossing. Although *E. crus-galli* is a self-compatible and highly autogamous species, accidental cross-pollination can happen by wind (Maun and Barrett, 1986). The potential long-range pollen dispersal mediated by wind can facilitate the recombination of different resistance genes evolved either in different individuals of the same population or in distant populations of the species. Under the high selective pressure imposed by recurrent same-herbicide use, these rare recombinants, quickly fixed by predominant self-pollination, can be at immediate advantage, thus, spreading into the local population in a few generations (Bracamonte et al., 2017; Gaines et al., 2020).

In summary, the first record of resistance to glyphosate was confirmed in different populations of *E. crus-galli* harvested in contrasting croplands of the Iberian Peninsula. The resistance levels depended on diverse NTSR mechanisms, but it also involves putative TSR ones, which were differentially stacked by populations in response to the massive selection caused by glyphosate and other factors. These results concluded that resistance was independent of climate, type of crop, and geographic region, and that the glyphosate resistance level observed on the different populations of *E. crus-galli* studied increased by the intense use of the herbicide. The quick selection of multiple resistance mechanisms to glyphosate, TSR and NTSR, including enhanced metabolism, is very worrying. Farmers must implement strategies of weed control, including available cultural and non-chemical strategies, as well as other herbicides with different modes of action to glyphosate in integrated weed management programs, to alleviate the herbicide selection pressure and suppress/reduce the evolution resistance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JGV-G and RDP: general idea and designed the experiments. JGV-G, JP, and RDP collected the different populations. JGV-G, AMR-D, JT, ID, JP, and RDP performed the research. RA-dlC, JT, ID, JP, and RDP analyzed and validated the results. All authors contributed to the article and approved the submitted version.

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frontiers in Plant Science

ORIGINAL RESEARCH
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First Case of Glyphosate Resistance in *Bromus catharticus* Vahl.: Examination of Endowing Resistance Mechanisms

Marcos Yanniccari[1,2], José G. Vázquez-García[3], María E. Gómez-Lobato[1,4], Antonia M. Rojano-Delgado[3], Pedro L. da C. A. Alves[5] and Rafael De Prado[3]*

[1]National Scientific and Technical Research Council, Buenos Aires, Argentina, [2]Chacra Experimental Integrada Barrow, MDA-INTA, Tres Arroyos, Argentina, [3]Department of Agricultural Chemistry and Edaphology, University of Cordoba, Cordoba, Spain, [4]Plant Physiology Institute (INFIVE), National University of La Plata, La Plata, Argentina, [5]School of Agricultural and Veterinarian Sciences, São Paulo State University (Unesp), Jaboticabal, Brazil

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Mahima Krishnan, University of Adelaide, Australia

***Correspondence:**
Marcos Yanniccari
marcosyanniccari@conicet.gov.ar

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Bromus catharticus Vahl. has been used as a valuable forage crop, but it has also been noted as a weed of winter crops and an invader in several countries. In Argentina, a putative glyphosate-resistant population of *B. catharticus* was identified as a consequence of the lack of effective control with glyphosate in the pre-sowing of wheat. Plant survival and shikimate accumulation analysis demonstrated a lower glyphosate-sensitivity of this population in comparison to a susceptible *B. catharticus* population. The resistant population was 4-fold more resistant to glyphosate than its susceptible counterpart. There was no evidence of target-site mechanisms of glyphosate resistance or an enhanced capacity to metabolize glyphosate in the resistant population. However, the resistant plants showed a lower foliar retention of glyphosate (138.34 μl solution g^{-1} dry weight vs. 390.79 μl solution g^{-1} dry weight), a reduced absorption of ^{14}C-glyphosate (54.18 vs. 73.56%) and lower translocation of ^{14}C-glyphosate from the labeled leaf (27.70 vs. 62.36%). As a result, susceptible plants accumulated a 4.1-fold higher concentration of ^{14}C-glyphosate in the roots compared to resistant plants. The current work describes the first worldwide case of glyphosate resistance in *B. catharticus*. A reduced foliar retention of herbicide, a differential rate of glyphosate entry into leaves and an altered glyphosate translocation pattern would be the most likely mechanisms of glyphosate exclusion.

Keywords: Brome, EPSPS gene, shikimate, glyphosate absorption, glyphosate translocation

INTRODUCTION

The genus *Bromus* L. comprises approximately 150 species distributed across temperate and cool regions of both hemispheres (Planchuelo and Peterson, 2000). Several species are used as natural pasture for grazing or have been introduced as forage in different countries (Planchuelo and Peterson, 2000). However, some *Bromus* species are aggressive

invaders posing enormous threats to native ecosystems (Bradford and Lauenroth, 2006; Speziale et al., 2014; Atkinson and Brown, 2016) or troublesome weeds on arable lands (Cussans et al., 1994; Andersson et al., 2002; Kleemann and Gill, 2006). Among them, *Bromus catharticus* Vahl. has been used as a valuable forage crop, but it has also been identified as a weed of winter crops and an invader in several countries (Casha et al., 2011; Ahumada and Troiani, 2016; Kovář, 2018).

Bromus catharticus originated in the Pampas of South America and was widely introduced into temperate regions worldwide (Planchuelo and Peterson, 2000; Planchuelo, 2006), but it also escaped into the wild in four continents (Dastgheib et al., 2003; Di Tomaso and Healy, 2007; Verloove, 2012; Muzafar et al., 2016; Bromilow, 2018). This is an annual, biennial, or perennial species and it shows two types of flowering, cleistogamic, and chasmogamic, but its reproductive behavior corresponds to that of an autogamous species with a low rate of allogamy (Naranjo, 1992; Gutierrez and Pensiero, 1998). Despite the high autogamy, morphologic and reproductive traits have shown plastic responses to environmental variations, explaining the greater adaptability of *B. catharticus* (Aulicino and Arturi, 2002).

This weed has shown a constancy (i.e., proportion of fields in which a given species is present) of around 20% in winter crops of central Pampas, Argentina (Poggio et al., 2004). In this region, *B. catharticus* starts the life cycle at midsummer and autumn (Ahumada and Troiani, 2016; Iroulart, 2020), when glyphosate is widely used for fallow weed control prior to sowing winter crops such as wheat and barley (Vigna et al., 2014). Biological characteristics, such as genetic plasticity, may predispose a weed species to evolve herbicide resistance (Moss et al., 2019). An herbicide treatment constitutes a challenging environment for a weed population, where an elimination of most local individuals occurs, but the adaptation from standing genetic variation allows the evolution of the population (Matzrafi et al., 2020). This process would be intensified when the weed is subjected to a widespread, persistent, and intense selection pressure with an herbicide (Powles and Yu, 2010).

In general terms, a resistant weed can survive to a normally lethal dose of herbicide by different mechanisms classified as target-site or non-target site resistance. The first category includes amino acid substitutions that affect herbicide interactions at the target enzyme and overexpression of the target site (Gaines et al., 2019). Non-target site mechanisms can be associated with the metabolism of the herbicide or exclusion of the herbicide from the target, either physically with enhanced cuticular and other structural barriers or physiologically with active vacuole sequestration, limited cellular uptake, or a rapid necrosis response (Sammons and Gaines, 2014; Ghanizadeh and Harrington, 2017).

In the south of Buenos Aires province, a population of *B. catharticus* was putatively identified as glyphosate-resistant based on the poor control at recommended doses of glyphosate (960 g ae ha^{-1}). As a consequence of the ineffective control in pre-sowing, *B. catharticus* becomes problematic weed species in wheat and barley crops, provoking yield losses of up to 70% (Iroulart, 2020). The aim of this work was to evaluate the magnitude of glyphosate resistance in the offspring of a putative glyphosate-resistant *B. catharticus*, and to determine the mechanisms associated with resistance to glyphosate in this weed species.

MATERIALS AND METHODS

Plant Material

In May 2017, 20 survived individuals of *B. catharticus* were collected from a fallow field (38.71°S and 60.48°W) where glyphosate at 960 g ae ha^{-1} had failed to control *B. catharticus*. In the last 8 years, the crop rotation involved wheat-soybean and barley-soybean, where weed control had been based on recurring applications of glyphosate in fallows and soybean crops, pinoxaden, dicamba, 2,4-D, and metsulfuron in wheat and barley crops.

The collected plants had 5–10 tillers and were dug from the field, taking care not to damage the root in the process. Immediately, the plants were transplanted into 2 L pots filled with soil (25% clay, 10% sand, and 4% organic matter) and placed outdoors in the Chacra Experimental Integrada Barrow (38.31°S and 60.23°W). At the end of the plant life cycle, all plants were harvested and manually threshed. Seeds of glyphosate-susceptible (S) *B. catharticus* were obtained from a population established as weed in the experimental station. Spikes of 20 susceptible plants were collected at random on January 2018. The seeds obtained were stored at room temperature until the beginning of the experiments.

For dose-response and shikimic accumulation assays, the plants were grown in 1 L pots filled with soil (25% clay, 10% sand, and 4% organic matter; one plant per pot) in a greenhouse at 21°C (average temperature) during the autumn season in the Chacra Experimental Integrada Barrow. The plants were irrigated according to water demand and avoiding water excess.

For foliar retention, absorption, translocation, metabolism, and enzyme activity assays, the plants were obtained as below: the seed were germinated in trays (15 cm× 15 cm× 8 cm) with peat moss substrate. The trays were taken to a growth chamber calibrated for 26/18°C day/night, 14 h photoperiod at 850 μmol^{-2} s^{-1} of light intensity, and 60% relative humidity. The seedlings germinated of both populations were transplanted into 250 ml (7 cm× 7 cm× 5 cm) pots (one plant $plot^{-1}$) with 230 g of substrate [soil:peat moss (1:1)]. The plants were taken to the greenhouse and irrigated daily.

Chemicals

A formulated glyphosate product (60.8% dimethyl amine salt of N-phosphomethyl glycine; Panzer® Gold, Argentina) was used in greenhouse tests and laboratory studies. Glyphosate, in analytical grade (>99%, Sigma-Aldrich, Madrid, Spain), was used to evaluate the biochemical and molecular aspects of glyphosate resistance. ^{14}C-glyphosate (glycine-2-^{14}C), with a radiochemical purity of 95% and specific activity 273.8 MBq $mmol^{-1}$, was obtained from the Institute of Isotopes Co., Ltd. (Budapest, Hungary).

Dose-Response Assays

The response of the putative glyphosate-resistant population of *B. catharticus* to glyphosate was compared to a susceptible population using dose-response experiments. A completely randomized design was used in order to test the sensitivity of the plants to glyphosate (60.8% dimethyl amine salt of N-phosphonomethyl glycine; Panzer® Gold, Argentina) doses of 0, 240 (only tested in the S population), 480, 960, 1920, 3,840, and 7,680 [only tested in the glyphosate-resistant (R) population] g ae ha^{-1}. The treatments were applied to plants with 2–3 tillers using a laboratory belt sprayer calibrated to deliver 200 L ha^{-1} (distilled water was used as carrier). There were 20 replicates for each herbicide rate, wherein each pot was a sampling unit.

Plant survival was recorded at 21 days after glyphosate treatment. Plants with severe visual injury (wilting, chlorosis of newly emerged leaves, and general browning) were recorded as "dead" plants, while "surviving" plants showed no apparent visual injury. The experiment was repeated twice.

Shikimic Acid Accumulation in Leaves

An experiment was carried out in order to determine the effects of the different doses of glyphosate on the accumulation of shikimate in leaves. R and S plants with 2–3 tillers were treated with glyphosate (60.8% dimethyl amine salt of N-phosphonomethyl glycine; Panzer® Gold, Argentina) at 0, 480, 960, and 1920 g ae ha^{-1}. A completely randomized design was used with five replicates per treatment. At 72 h after treatment (HAT), 0.05 g of fresh weight from the middle third of the youngest fully expanded leaf of each replicate was used for shikimic acid determination, following the methodology described by Perez-Jones et al. (2007). Shikimic acid was quantified with a spectrophotometer (Numak 752 UV-Vis) at 382 nm. The determination of the concentration of shikimic acid was based on a shikimate (3a,4a,5b-trihydroxy-1-cyclohexene-1-carboxylic acid, 99%. Sigma Aldrich, Inc.) standard curve. The experiment was repeated twice.

Glyphosate Foliar Retention

The methodology used for the foliar retention was described by Palma-Bautista et al. (2020) with some modifications and carried out at the University of Córdoba (Spain). Young plants with 4–6 leaves of R and S *B. catharticus* populations were sprayed with 360 g ae ha^{-1} of glyphosate and 100 mg L^{-1} Na-fluorescein using a laboratory system (SBS-060 De Vries Manufacturing, Hollandale, MN, United States) equipped with 8002 flat fan nozzles delivering 200 L ha^{-1} at 250 kPa at the height of 50 cm from plant level. When plants dried (40–60 min), each shoot tissue was cut at ground level. The tissue was submerged in test tubes with 50 ml of 5 mM NaOH for 30 s to remove the spray solution. The washing solution was recovered in glass flasks. Fluorescein absorbance was determined using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) with an excitation wavelength of 490 nm and an absorbance wavelength at 510 nm. Then, the plants were wrapped in filter paper and oven dried at 80°C for 48 h and weighed. The experiment was laid out in a completely randomized design with 10 replicates. It was repeated twice, and the results as microliter of sprayed solution retained per g dry weight were combined for analysis.

^{14}C-Glyphosate Absorption, Translocation, and Visualization

^{14}C-glyphosate (glycine-2-^{14}C) plus commercial glyphosate solution was applied to R and S *B. catharticus* plants following the methods described by Vázquez-García et al. (2020a,b) and carried out at University of Córdoba (Spain). Plants were treated at the 3–4-leaf stage and there were five repetitions and each experiment was arranged in a completely randomized design.

The second leaf was marked and covered with aluminum foil before spraying the whole plant with 360 g ae ha^{-1} glyphosate, and 30 min later the aluminum foil was removed. The final glyphosate concentration corresponded to 360 g ae ha^{-1} in 200 L ha^{-1}, which contained a specific activity of 100,000 dpm μl^{-1} (equivalent to 1.667 kBq μl^{-1}). Five plants per population were treated with one drop (1 µl $plant^{-1}$) of the solution on the adaxial surface of the second leaf. After treatment, the plants were maintained in the growth chamber at the growing conditions described in section Plant Material.

The absorbed ^{14}C-glyphosate was removed from the treated leaves (at 12, 24, 48, 72, 96, and 120 HAT) by washing three times separately with 1 ml of a water-acetone solution (1:1 v/v) each time. The washing solution was mixed with 2 ml of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard, MA, United States) and analyzed by liquid scintillation spectrometry (LSS) using a scintillation counter (LS 6500, Beckman Coulter Inc., Fullerton, CA, United States) with reading time of 10 min per sample.

After washing, whole plants were removed from the pot and sectioned into treated leaves, the remainder of the shoot and the roots (this plant section was carefully washed with distilled water and excess moisture removed with paper towel). The samples were stored in cellulose cones (Perkin-Elmer, BV BioScience Packard, MA, United States), dried in an oven at 60°C for 96 h, and combusted in a biological oxidizer (Packard Tri Carb 307, Packard Instrument Co., Downers Grove, IL, United States). The CO_2 released from the combustion was captured in 18 ml of a mix of Carbo-Sorb E and Permafluor (1:1 v/v; Perkin-Elmer, BV BioScience Packard, MA, United States). The radioactivity in dpm of each individual sample was quantified by LSS over a 10 min period per sample. The radioactive values of absorption and translocation of ^{14}C were expressed as a percentage of the total ^{14}C-herbicide applied and absorbed, respectively.

To visualize the translocation of ^{14}C-glyphosate, three plants were treated under the same conditions as in the previous assay. At 96 and 120 HAT, plants were washed individually, fixed on filter paper, and dried at 25°C (room temperature) for 1 week. The plants were pressed for 4 h under a phosphor store film (Storage Phosphor System: Cyclone, Perkin-Elmer Packard BioScience BV, MA, United States) and visualized using a phosphor imager Cyclone (Perkin-Elmer, Packard BioScience BV, MA, United States).

Glyphosate Metabolism

For this assay, plants were treated with a glyphosate dose of 360 g ae ha^{-1} when they were at the 3 to 4-leaf stage, following the procedure and equipment used in the glyphosate foliar retention assay. The same numbers of plants, without glyphosate treatment, were used as blank. Plants were cut at 120 HAT, washed with distilled water (to remove excess herbicide on the surface of the leaf), and dried. Rapidly, they were frozen by liquid nitrogen and stored at a temperature less than or equal to −40°C before being used. For the determination and quantification of glyphosate and its metabolites metabolites [amino methyl phosphonic acid (AMPA), glyoxylate, sarcosine, and formaldehyde], the methodology described by Rojano-Delgado et al. (2010) was followed, using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD, wavelength range 190–600 nm). The used background electrolyte was an aqueous solution at pH 7.5, containing 10 mM potassium phthalate, 0.5 mM hexadecyltrimethylammonium bromide (CTAB), and 10% acetonitrile. The calibration equations were obtained using standards of known concentration of glyphosate and metabolites were supplied by Sigma-Aldrich (St. Louis, MI). The experiment was arranged in a completely randomized design with five replications (individual plants) per population and treatment and repeated three times.

EPSPS Basal Activity and Dose-Response

Five grams of leaf tissue from R and S plants, finely powdered, were transferred to tubes with 100 ml of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine), 70 µl of β-mercaptoethanol and 1% polyvinylpolypyrrolidone (PVPP). Enzyme extraction was performed following the protocol described by Sammons et al. (2007).

The specific 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity was assayed in the presence of glyphosate (>99%) at different concentrations (from 0 to 5,000 µM) using the EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, United States). The EPSPS enzyme reaction substrates were phosphoenolpyruvate and shikimate-3-phosphate, which were supplied by Sigma-Aldrich (Madrid, Spain). The release of phosphate was measured for 10 min at 360 nm in a spectrophotometer (model DU-640, Beckman Instruments Inc., Fullerton, United States). The total soluble protein (TSP) in the extract was measured using a Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain), following the manufacturer's instructions. The EPSPS activity was measured for 10 min at 360 nm in a spectrophotometer (model DU-640) to determine the amount of inorganic phosphate (µmol) released per µg of TSP per min (µmol Pi μg^{-1} TSP min^{-1}). The EPSPS activity was expressed as a percentage relative to the control (absence of glyphosate). Three technical replications of each glyphosate concentration were analyzed per population. The experiment was repeated twice.

EPSPS Gene Sequencing

Total DNA was extracted from the leaf tissue of five R plants (survivors at a glyphosate dose of 1,920 g ae ha^{-1}), following the protocol of Doyle and Doyle (1990). DNA yield and quality were evaluated spectrophotometrically. The DNA was used as a template to amplify the EPSPS sequence. The forward primer (5'-AGCTGTAGTCGTTGGCTGTG-3') and reverse primer (5'-GCCAAGAAATAGCTCGCACT-3') were employed to amplify a highly conserved region encompassing the positions of all the known mutations that confer glyphosate resistance (Sammons and Gaines, 2014). A 1,395-bp fragment was obtained in the PCR reactions (initial denaturation at 94°C for 2 min and 30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min), containing: 300 ng DNA template, 0.8 µM of each primer, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$, 1X reaction buffer (Inbio Highway), and 1 U Taq polymerase (Inbio Highway) in a 25 µl reaction mix.

PCR products were purified and sequenced from both ends through Macrogen service (Macrogen Inc., Seoul, South Korea). The sequence data obtained were cleaned, aligned, and compared at 101, 102, 106, 144, and 192 codons (numbers based on the plant EPSPS numbering system used by Padgette et al., 1996) using BLAST of the National Center for Biotechnology Information (NCBI).

Statistical Analysis

Survival and EPSPS activity data were used to build dose-response curves with a non-linear log-logistic regression model as described by Streibig et al. (1993):

$$y = c + \{(d - c) / \left[1 + (x / g)^b\right]\}$$

In this equation, y represents the percentage of response at the herbicide rate x; c and d are the lower (fixed at 0 for LD_{50}) and upper asymptote, respectively; b is the slope of the line at g; and g is the herbicide concentration required to reach 50% of the maximum response for EPSPS enzyme activity (I_{50}) or the glyphosate dose causing 50% mortality (LD_{50}). To assess the accuracy of the models, F-test for model significance, residual variance analysis, and coefficient of determination (R^2) were calculated. LD_{50} and I_{50} values from resistant and susceptible populations were compared with the F-test ($p < 0.05$; GraphPad Prism 6 Software) and a resistance index (RI) was calculated as the ratio of the LD_{50} of the resistant population compared to the susceptible population.

An ANOVA was performed to evaluate the differences between populations and treatments. The differences between the mean values of shikimic acid contents, glyphosate foliar retention, and ^{14}C-glyphosate absorption and translocation were compared with Fisher test ($p < 0.05$; Statistica® v7.1. Stat Soft).

RESULTS

Glyphosate-Sensitivity: Plant Survival and Shikimate Accumulation

At least 50% of plants from the R population survived to 1 and 2-fold of the recommended dose of glyphosate (960 g ae ha^{-1}); while none of the S plants survived these treatments (**Figure 1**).

Regression models fitted to plant survival of both populations were compared, and LD_{50} parameters differed significantly between S and R populations ($p = 0.004$). The LD_{50} calculated for the R population was higher than the recommended dose of glyphosate (1750 vs. 960 g ae ha^{-1}) and the RI achieved was 4.0.

The response of shikimate accumulation to the glyphosate dose was significantly different between populations ($p < 0.01$; **Figure 2**). While the basal content of shikimate was similar in S and R plants, a significant shikimate accumulation of 2.9 and 6.0-fold was detected in S plants when treated with 960 and 1920 g ae ha^{-1}, respectively. In contrast, R plants showed no significant changes in shikimate concentration among the different treatments (**Figure 2**).

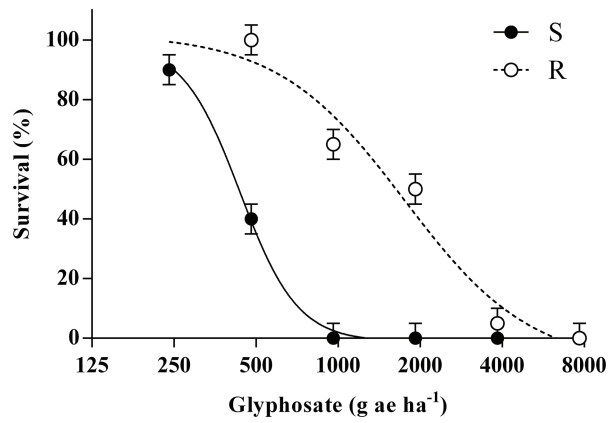

FIGURE 1 | Effects of glyphosate doses on plant survival for the glyphosate-susceptible (S) and -resistant (R) *Bromus catharticus* at 21 days after treatment. Symbols represent mean values and bars indicate ± 1 SEM. The predicted responses are shown by lines according to the adjusted models: (S) $y = -1+(99+1)/[1+(x/436)^{4.1}]$; $p < 0.01$; $R^2 = 0.99$ and (R) $y = -9+(102+9)/[1+(x/1750)^{1.8}]$; $p < 0.01$; $R^2 = .97$.

Glyphosate Foliar Retention

S and R plants of *B. catharticus* treated with glyphosate showed different foliar retention of the herbicide. S leaf retention was 390.79 ± 49.10 (SE; µl solution g^{-1} dry weight) while the R population had a lower value of 138.34 ± 22.36 (SE; µl solution g^{-1} dry weight). Foliar retention capacity was 2.83 times greater in the S population as compared to the R population (**Figure 3**).

^{14}C-Glyphosate Absorption, Translocation, and Visualization

Total recovery of ^{14}C-glyphosate in this research was 94.3 ± 2.1 (SE) % and 95.1 ± 1.4 (SE) % for R and S populations of *B. catharticus*, respectively. Absorption of ^{14}C-glyphosate was slow in both R and S populations until 48 HAT (**Figure 4A**). At this time, the S population had absorbed 21.96 ± 1.81 (SE) % glyphosate, while the R population had only absorbed 17.86 ± 1.47 (SE) %. From 48 HAT, the absorption began to be exponentially more pronounced in the S population. The maximum absorption rate of glyphosate was observed at 120 HAT, which was 1.4-fold higher in the S population [73.56 ± 2.40 (SE) %] than in the R population [54.18 ± 4.94 (SE) %]. At any time, compared with the R plants, S plants translocated more ^{14}C-glyphosate (as a percentage of that absorbed) from the treated leaves to the rest of the plant and roots (**Figures 4B–D**). The corresponding accumulation of ^{14}C-glyphosate was measured in the remaining shoot tissue (rest of plants) was greater for the S population as compared to R counterparts (**Figure 4C**). Differences in accumulation of ^{14}C-glyphosate in roots between S and R populations were most noticeable at times later than 48 HAT. The S vs. R plants demonstrated a 4.1-fold higher root concentration of ^{14}C-glyphosate at 120 HAT (**Figure 4D**).

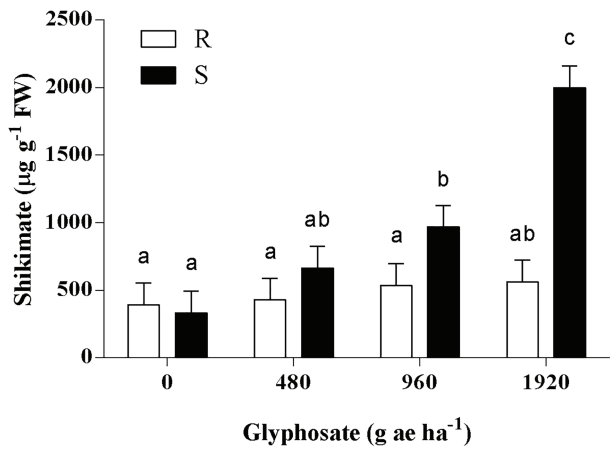

FIGURE 2 | Accumulation of shikimic acid in the last expanded leaf of glyphosate-susceptible (S) and -resistant (R) *Bromus catharticus* at 72 h after glyphosate treatment. Columns represent mean values, and vertical bars indicate the SEM. Letters above the bars indicate statistical significance ($p < 0.05$).

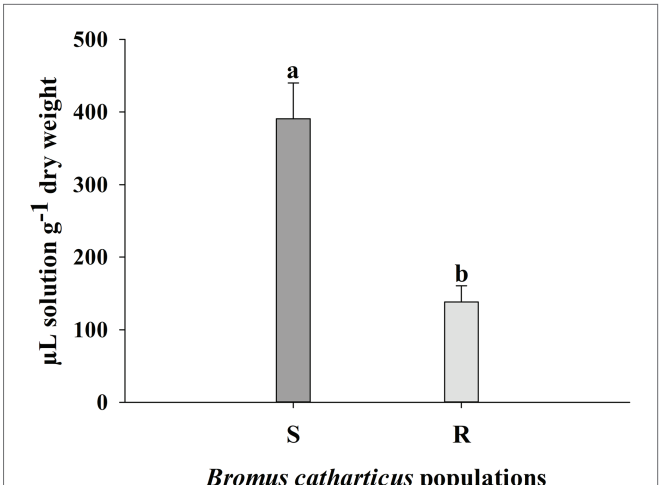

FIGURE 3 | Spray retention of a glyphosate solution by resistant (R) and susceptible (S) *Bromus catharticus* plants. Error bars are the SEM. Different letters above the bars indicate significant differences between populations ($p < 0.05$).

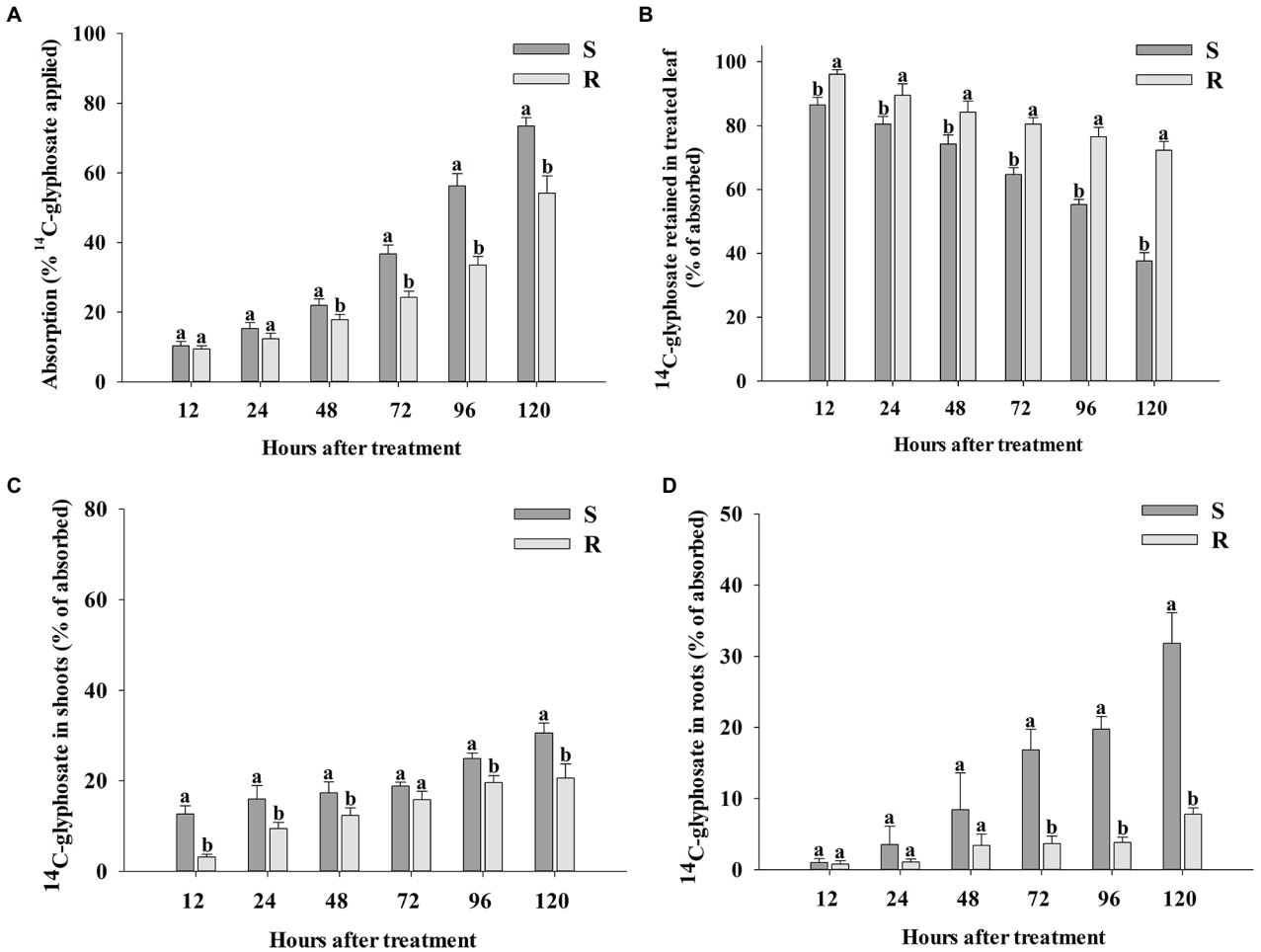

FIGURE 4 | Percentage of absorption of ^{14}C-glyphosate **(A)** in glyphosate-susceptible (S) and -resistant (R) *Bromus catharticus*, ^{14}C-glyphosate detected in the labeled leaf **(B)** and translocation from treated leaf to rest of plants **(C)** and the root system **(D)** at 12–120 HAT. Error bars are the SEM per time evaluated. Different letters above the bars indicate statistical differences between populations at the same time of evaluation ($p < 0.05$).

The Phosphor Imager images shown confirmed the previous results obtained with the LSS in absorption and translocation assays (**Figure 5**). At 96 and 120 HAT, the plants of the R population absorbed and translocated smaller amounts of ^{14}C-glyphosate from the treated leaf to the root than the S plants (**Figure 4**).

Glyphosate Metabolism

The data obtained in this study (**Table 1**) showed that there is no glyphosate metabolism. In fact, only glyphosate and a minimum amount of glyoxylate can be observed at 120 HAT. The latter cannot be considered a metabolite of glyphosate because its origin is not only glyphosate; therefore, if AMPA does not appear (for example), it cannot be considered as a metabolite of this herbicide.

EPSPS Basal Activity and I_{50} Values

No differences were observed between R and S populations in respect to the concentration of glyphosate required to inhibit EPSPS activity by 50% (I_{50}), being 0.150 and 0.120 μM of glyphosate, respectively (**Figure 6**). In addition, the EPSPS activity in the absence of glyphosate was similar in both R and S populations, being 1.82 ± 0.03 (SE) and 1.45 ± 0.03 (SE) μmol Pi μg^{-1} TSP min^{-1}, respectively. In this case, no differences were apparent between the S and R plants for either EPSPS activity in the absence of glyphosate or the inhibition response to glyphosate (I_{50}).

EPSPS Gene Sequencing

The fragment sequenced (MT454262) included exons (part of 2, 3, and 4) and introns (2, 3, and part of 4) according to the structure described by Aramrak et al. (2015) The sequence of *B. catharticus* showed around 86% of identity with the three genomic copies of *EPSPS* of *T. aestivum* on chromosomes 4A, 7A, and 7D (KP411547.1, KP411548.1, and KP411549.1). However, the exons were more highly conserved than introns, where the translated regions showed at least 90% of identity with the three genes of *T. aestivum*.

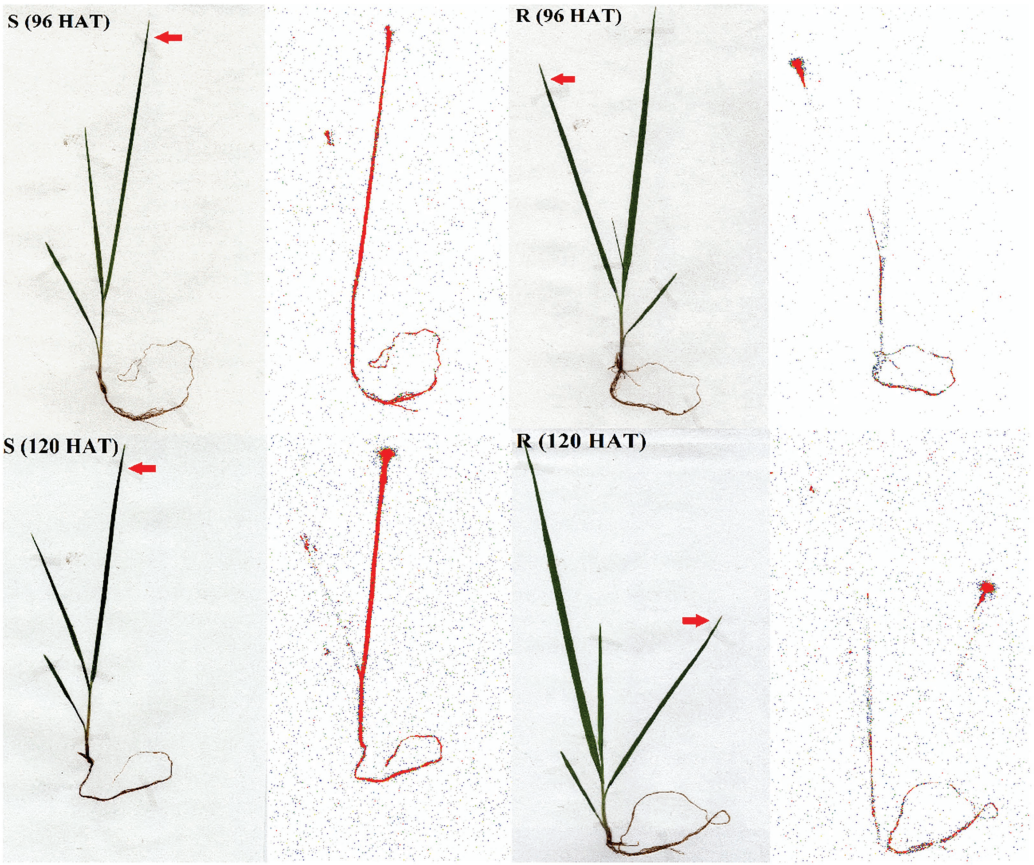

FIGURE 5 | Digital images and ^{14}C-glyphosate visualization in glyphosate-susceptible (S) and -resistant (R) *Bromus catharticus* populations. The translocation visualization was obtained from treated plants at 96 and 120 HAT. Arrows indicate the treated leaves. The concentration of ^{14}C-glyphosate is highlighted in red.

TABLE 1 | Glyphosate metabolism (%) at 120 h after treatment (HAT) in glyphosate-susceptible (S) and -resistant (R) *Bromus catharticus* populations. Mean values ± SE are shown.

	Metabolism (%) at 120 HAT		
B. catharticus	**Glyphosate**	**AMPA**	**Glyoxylate**
S	97.48 ± 3.18	–	2.81 ± 0.82
R	96.92 ± 2.21	–	3.05 ± 0.49

Analyzing the differences between exons of *B. catharticus* vs. *T. aestivum*, most nucleotide mismatches were associated with silent substitutions, but seven mismatches involved six codon changes: Gly-134-Ala, Gln-141-Lys, Asp-146-Thr, Asn-154-Asp, Lys-163-Thr, and Glu-194-Gly. However, no substitutions were recorded at codons 101, 102, 103, 106, 144, and 192, which have been associated with low glyphosate-sensitivity.

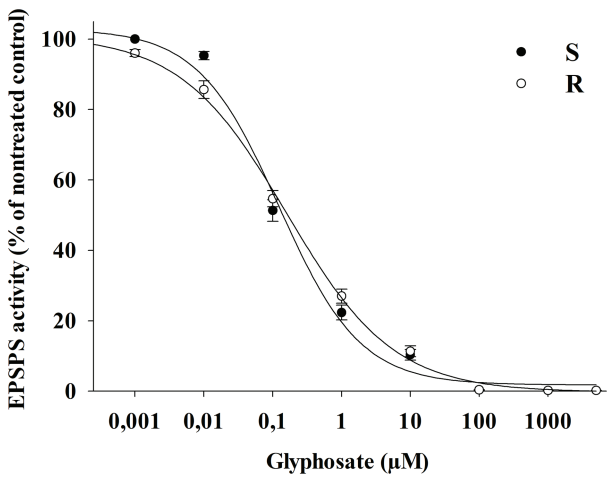

FIGURE 6 | EPSPS enzyme activity of glyphosate-susceptible (S) and -resistant (R) *Bromus catharticus* populations exposed to different glyphosate concentrations (µM), expressed as a percentage of the untreated control. The predicted responses are shown by lines according to the adjusted models: (S) $y = (103)/[1+(x/0.12)^{0.73}]$; $p < 0.01$; $R^2 = 0.99$ and (R) $y = (101)/[1+(x/0.16)^{0.56}]$; $p < 0.01$; $R^2 = 0.99$.

Discussion

Within the genus *Bromus*, populations of *B. diandrus*, *B. sterilis*, and *B. rubens* have evolved glyphosate resistance in Australia, United Kingdom, and Spain, respectively (Malone et al., 2016;

Davies et al., 2019; Heap, 2020), and *EPSPS* gene amplification was reported as the mechanism of glyphosate resistance in *B. diandrus* (Malone et al., 2016). Until now, no antecedents of glyphosate-resistance were detected in American species of *Bromus*.

In the current work, the plant survival analysis showed that the glyphosate-sensitivity of the R *B. catharticus* population was around 4-fold lower than the S population (**Figure 1**). Consistent with this finding, the shikimate content in the leaves of R plants showed no significant changes in response to glyphosate treatments (**Figure 2**). These results support an inherited ability of plants from the R population to survive and reproduce after a normally lethal dose of glyphosate. All surviving plants produced viable seeds, so in that sense, the progeny obtained from individuals treated to 1,920 g ae ha^{-1} was used to determine the mechanism of resistance.

No evidence was found for target-site mechanisms of resistance to glyphosate in the R population. Neither a target-site mutation nor differences in basal EPSPS activity were identified between both populations. However, our results revealed that R plants retained just under half of the amount quantified in S plants (**Figure 3**) but spraying a double dose of glyphosate on the R population was not enough to match the mortality of the S population (**Figure 1**). A lower foliar retention of herbicide would constitute the first barrier for glyphosate efficacy because it limits the amount that can subsequently enter the plant. In any case, a differential glyphosate foliar retention has not been detected as a strong mechanism of resistance *per se*, which instead has been linked to an altered glyphosate uptake and translocation or even duplication of *EPSPS* gene copies in glyphosate-resistant *Lolium multiflorum* populations (Michitte et al., 2007; Fernández-Moreno et al., 2017). In the *B. catharticus*, population analyzed important mechanism of herbicide selectivity would be associated with other exclusion mechanisms as in the cases quoted above.

No evidence was obtained for an enhanced capacity to metabolize glyphosate during the time of evaluation (up to 120 HAT; **Table 1**), and this observation supports the tracking of glyphosate movement in order to confirm differences in herbicide uptake and translocation between populations. Comparing the glyphosate absorption process in both populations, R plants showed a slower uptake from 48 to 120 HAT. Thus, the maximum absorption rate of glyphosate was detected at 120 HAT, which was 25% lower in the R population in respect to the glyphosate uptake quantified in S plants (**Figure 4A**). A differential rate of glyphosate entry into leaves has been detected in other weed species, where R accessions showed up to 40% of reduction in the absorption of herbicide; however, this trait seems to be frequently associated with an altered pattern translocation (Michitte et al., 2007; de Carvalho et al., 2012; Vila-Aiub et al., 2012; Dominguez-Valenzuela et al., 2017; Palma-Bautista et al., 2019).

Notwithstanding differences in glyphosate absorption between populations, the current results show that herbicide uptake was around 10–15% of glyphosate applied at 12 and 24 HAT and no differences were detected between R and S plants during this period (**Figure 4A**). Nevertheless, the glyphosate translocation from the labeled leaf was significantly different between both populations at 12 and 24 HAT (**Figures 4B,C**). Throughout the entire analysis period, R plants translocate half or less of the glyphosate absorbed compared to S plants (**Figure 4B**). As a consequence, the translocation of glyphosate to the roots was four times greater in S plants as compared to R counterparts at the last moment of evaluation (**Figure 4D**). This evidence suggests that impaired translocation of glyphosate would be the primary mechanism of resistance in the R *B. catharticus* population.

The reduction in glyphosate movement to sensitive tissues, such as shoots and root meristems, would have a large effect on plant survival (Preston and Wakelin, 2008; Shaner, 2009). Since the herbicide affects actively growing tissues, the demand for assimilates would decrease and consequently induce an accumulation of carbohydrates in the leaves associated with a feedback inhibition of CO_2 fixation, but as the light stage of photosynthesis is initially unaffected, a redirection of electrons to alternative electron sinks occurs, conducive to oxidative stress, that ultimately leads to plant death (Yanniccari et al., 2012a,b). As a consequence, a restricted translocation of glyphosate has been frequently detected as an important mechanism of resistance in several weed species (Feng et al., 2004; Wakelin et al., 2004; Koger and Reddy, 2005; Vila-Aiub et al., 2012; Ghanizadeh et al., 2016; Dominguez-Valenzuela et al., 2017; Vázquez-García et al., 2020a,b). Going further, a process of glyphosate sequestration within the cell vacuole was detected as a basis of glyphosate altered translocation in resistant *Conyza canadensis* and *Lolium* spp. populations (Ge et al., 2010, 2012, 2014).

Bromus spp. weeds have emerged as a major challenge for arable farmers because there is no effective herbicide for its control in cereals (Dastgheib et al., 2003). In that sense, *B. catharticus* should be controlled during the fallow period prior to sowing winter cereals, for which glyphosate is widely used in Argentina (Vigna et al., 2014). The current work shows the first worldwide case of glyphosate resistance in *B. catharticus*, where an altered glyphosate translocation pattern was revealed from 12 HAT onwards and this represented to be the primarily mechanism of resistance. However, a lower foliar retention and a reduced absorption of glyphosate were also evidenced as barriers of glyphosate exclusion mechanisms. Given the relevance of genetic factors in the dynamics of herbicide-resistance (Ghanizadeh et al., 2019), it is important to know the basis of the inheritance of glyphosate resistance in *B. catharticus*. Preventing the spread of this trait is a major challenge for future studies, considering the high capacity of adaptation of this weed to a wide range of habitats and growing conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, MT454262.

AUTHOR CONTRIBUTIONS

MY, JV-G, PA, and RP designed experiments. MY, JV-G, AR-D, and MG-L performed the experiments and data analysis. MY, JV-G, AR-D, PA, and RP wrote the manuscript. All authors have reviewed and approved the final manuscript.

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Non-target Site Herbicide Resistance Is Conferred by Two Distinct Mechanisms in Black-Grass (*Alopecurus myosuroides*)

*Sara Franco-Ortega, Alina Goldberg-Cavalleri, Andrew Walker, Melissa Brazier-Hicks†, Nawaporn Onkokesung and Robert Edwards**

Agriculture, School of Natural, and Environmental Science, Newcastle University, Newcastle upon Tyne, United Kingdom

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***Correspondence:**
Robert Edwards
robert.edwards@newcastle.ac.uk

†Present address:
Melissa Brazier-Hicks, Syngenta Jeallott's Hill International Research Centre, Bracknell, United Kingdom

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Non-target site resistance (NTSR) to herbicides in black-grass (*Alopecurus myosuroides*) results in enhanced tolerance to multiple chemistries and is widespread in Northern Europe. To help define the underpinning mechanisms of resistance, global transcriptome and biochemical analysis have been used to phenotype three NTSR black-grass populations. These comprised NTSR1 black-grass from the classic Peldon field population, which shows broad-ranging resistance to post-emergence herbicides; NTSR2 derived from herbicide-sensitive (HS) plants repeatedly selected for tolerance to pendimethalin; and NTSR3 selected from HS plants for resistance to fenoxaprop-*P*-ethyl. NTSR in weeds is commonly associated with enhanced herbicide metabolism catalyzed by glutathione transferases (GSTs) and cytochromes P450 (CYPs). As such, the NTSR populations were assessed for their ability to detoxify chlorotoluron, which is detoxified by CYPs and fenoxaprop-P-ethyl, which is acted on by GSTs. As compared with HS plants, enhanced metabolism toward both herbicides was determined in the NTSR1 and NTSR2 populations. In contrast, the NTSR3 plants showed no increased detoxification capacity, demonstrating that resistance in this population was not due to enhanced metabolism. All resistant populations showed increased levels of *Am*GSTF1, a protein functionally linked to NTSR and enhanced herbicide metabolism. Enhanced *Am*GSTF1 was associated with increased levels of the associated transcripts in the NTSR1 and NTSR2 plants, but not in NTSR3, suggestive of both pre- and post-transcriptional regulation. The related HS, NTSR2, and NTSR3 plants were subject to global transcriptome sequencing and weighted gene co-expression network analysis to identify modules of genes with coupled regulatory functions. In the NTSR2 plants, modules linked to detoxification were identified, with many similarities to the transcriptome of NTSR1 black-grass. Critical detoxification genes included members of the CYP81A family and tau and phi class GSTs. The NTSR2 transcriptome also showed network similarities to other (a)biotic stresses of plants and multidrug resistance in humans. In contrast, completely different gene networks were activated in the NTSR3 plants, showing similarity to the responses to cold, osmotic shock and fungal infection determined in cereals. Our results demonstrate that NTSR in black-grass can arise from at least two distinct mechanisms, each involving complex changes in gene regulatory networks.

Keywords: herbicide metabolism, pendimethalin, fenoxaprop, xenome, black-grass, weighted gene co-expression network analysis (WGCNA), non-target site resistance (NTSR)

INTRODUCTION

Black-grass (*Alopecurus myosuroides*) is an annual grass weed of cereals that is widely dispersed in genetically diverse populations across Western Europe (Moss, 1979). Herbicide resistance is now widespread in these populations; and in the United Kingdom, the respective loss of weed control incurs an economic cost of ∼0.5 bn GBP/year, being associated with 1 million ton/year of yield loss in wheat production (Varah et al., 2020).

Within these resistant populations, non-target site resistance (NTSR) is commonly encountered and particularly difficult to combat, as it contributes to loss of control of many pre- and post-emergence selective herbicides, irrespective of their chemistry or mode of action (Preston, 2004). NTSR is a complex, multigenic trait that invokes diverse resistance mechanisms to herbicides and is linked to their reduced uptake, translocation, and enhanced detoxification, as well as less-well-understood broad-ranging cytoprotective mechanism (Gaines et al., 2010; Délye, 2013). Elevated herbicide detoxification, which is also known as enhanced metabolic resistance (EMR), is one of the best studied mechanisms of NTSR in wild grasses, including rigid ryegrass (*Lolium rigidum*), wild oat (*Avena fatua*), and black-grass (Délye et al., 2011; Délye, 2013). Central to EMR is the enhanced expression of proteins involved in herbicide detoxification, which includes cytochromes P450 (CYPs), glutathione transferases (GSTs), UDP-glycosyltransferases (UGTs), and ATP-binding cassette transporters (ABC transporters). These proteins act collectively to detoxify herbicides (Délye, 2013). In contrast to EMR, the molecular components of other NTSR mechanisms remain largely unknown.

In a previous study, using quantitative proteomics (Tétard-Jones et al., 2018), we identified three "types" of NTSR, namely, (1) multiple resistance to herbicides with differing modes of action (NTSR1), (2) cross-resistance to chemistries acting on the same mode of action (NTSR2), and (3) resistance to a specific herbicide chemistry (NTSR3). The classification of these overall NTSR types was made possible by characterizing populations that had been selected for through repeated selections with specific herbicides. The NTSR1 plants were derived from the field-derived Peldon population that had evolved resistance over generations of exposure to multiple herbicide classes. The other types of NTSR black-grass plants were derived from the herbicide-sensitive (HS) Rothamsted population, a lineage of black-grass that has never been exposed to herbicides. The NTSR2 plants were generated by a repeated selection with the pre-emergence herbicide pendimethalin, whereas the consecutive selection of the same HS plants for resistance using the post-emergence herbicide fenoxaprop-*P*-ethyl yielded a NTSR3 population. Previous studies have shown that elevated levels of the phi (F) class GST *Am*GSTF1 were integrally linked to NTSR in black-grass (Cummins et al., 2013), with proteomics showing enhanced levels of the protein in resistant populations (Tétard-Jones et al., 2018). Proteomics also demonstrated that additional proteins were induced in the NTSR1 and NTSR2 plants that differed from those in the NTSR3 population (Tétard-Jones et al., 2018). Cumulatively, these results indicate the presence of unknown NTSR mechanisms in these black-grass populations.

To explore these NTSR mechanisms in greater detail and to reduce the effects of background genetic diversity in differing black-grass populations, we have compared the global transcript expression profiles of the NTSR2 and NTSR3 plants with those in the parent HS populations using a tiered approach (**Figure 1**). In particular, we have applied a weighted gene co-expression network analysis (WGCNA), a powerful tool to study regulatory transcriptional networks within transcriptome datasets, which also helps identify key genes that underpin core mechanisms (Zhang and Horvath, 2005; Horvath, 2011). In the current study, WGCNA subdivides interconnected genes into modules that can be correlated with the differing types of NTSR. Core to our analysis, we have performed WGCNA on the related

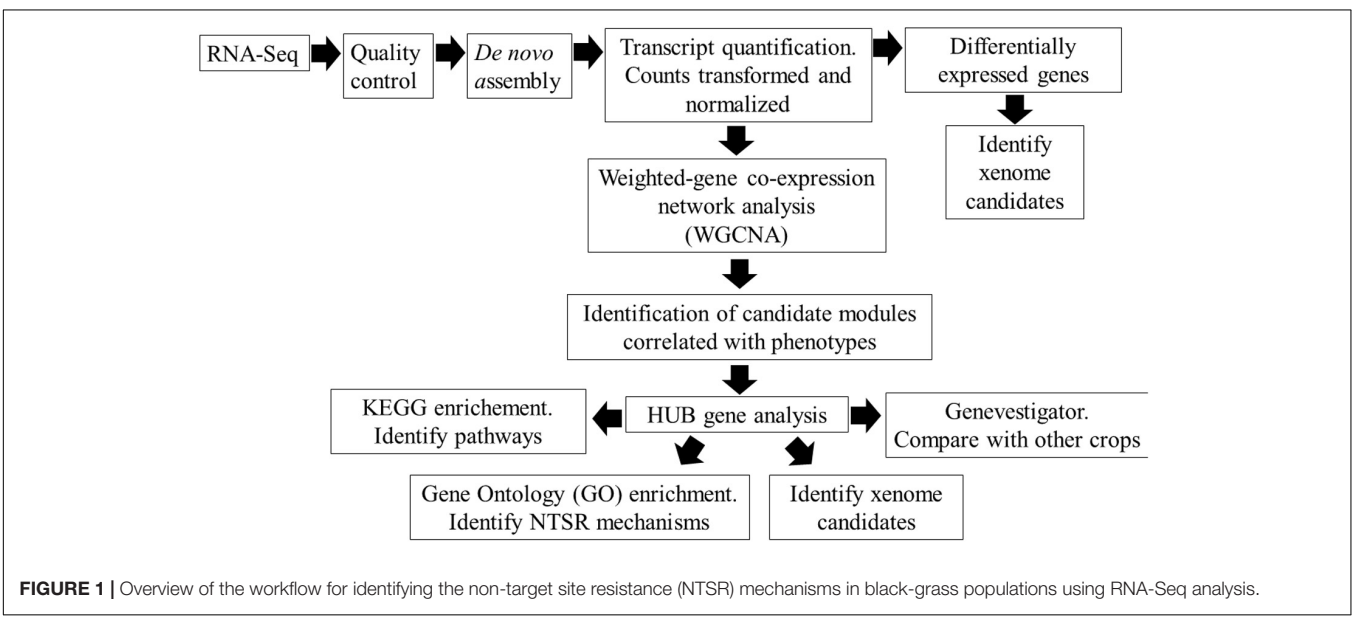

FIGURE 1 | Overview of the workflow for identifying the non-target site resistance (NTSR) mechanisms in black-grass populations using RNA-Seq analysis.

HS, NTSR2, and NTSR3 plants and compared the differentially regulated genes to those present in the field-derived Peldon NTSR1 population, allowing us to compare the transcriptomes of all three NTSR subtypes. In each case, we have determined changes in latent gene expression in each population of resistant black-grass and have not taken into account the active response of the plants to herbicide exposure. While the induction and suppression of specific genes in response to chemical stress would have been insightful, the differences in resistance profile to the multiple herbicides used in the study rendered this approach impractical.

MATERIALS AND METHODS

Black-Grass Populations and Plant Growth Conditions

The NTSR2 and NTSR3 plants were generated from the HS population by selecting for survivors from repeated field-rate applications of pendimethalin (×8) and fenoxaprop-*P*-ethyl (×6), respectively, over consecutive growing seasons. The resulting NTSR2 and NTSR3 plants were assessed for their tolerance to herbicides acting on acetyl-CoA carboxylase (ACCase), acetolactate synthase (ALS), tubulin assembly, and fatty acid elongation, respectively (Marshall et al., 2013). The NTSR3 plants were only resistant toward the ACCase herbicide fenoxaprop-*P*-ethyl, while the NTSR2 population was resistant toward pendimethalin (inhibitor of tubulin assembly) and ACCase inhibitors (including fenoxaprop-*P*-ethyl), but not to compounds acting on ALS (Tétard-Jones et al., 2018). Neither of the resistant populations showed evidence of TSR-linked mutations in the respective ACCase or ALS genes (Marshall et al., 2013). The field-derived Peldon plants were used as a well-characterized reference NTSR1 black-grass population demonstrating EMR and showing cross-resistance to ACCase, tubulin assembly, and photosystem II inhibitors including fenoxaprop-*P*-ethyl, diclofop-methyl fenoxaprop-*P*-ethyl, fluazifop-*P*-butyl, tralkoxydim, cycloxydim, pendimethalin, and chlorotoluron (Hall et al., 1997; Marshall et al., 2013). Peldon plants are also reported to show insensitivity to ALS inhibitors such as iodosulfuron and mesosulfuron due to mutations in the targeted ALS gene (Marshall and Moss, 2008).

Black-grass seeds were pre-germinated in petri dishes on four layers of filter paper (Whatman No. 1, Sigma Aldrich, Gillingham, United Kingdom) wetted with 7 ml of sterile deionized water. Petri dishes were maintained at 4°C in the dark for 7 days prior to transfer to a growth cabinet with a 16 h light/8 h dark with intensity of 220 μmol m^{-2} s^{-1} and 18°C/16°C temperature cycle. For each population, five seedlings were sown into 10 cm plastic pots (n = 5) containing John Innes No. 2 compost (East Riding Horticulture, York, United Kingdom), mixed with Osmocote slow release fertilizer (Osmocote, Suffolk, United Kingdom), and propagated in a growth cabinet (Sanyo, MLR-351 SANYO Electric Co., Ltd., Osaka, Japan).

Herbicide Metabolism Assays

Metabolism studies in black-grass were conducted using herbicides undergoing primary detoxification by either CYPs (the phenylurea chlorotoluron) or GSTs (the aryloxyphenoxypropionate fenoxaprop acid). Leaves from the HS, NTSR1, NTSR2, and NTSR3 plants, each at the two-tiller stage (4 weeks), were cut into 1 cm-long pieces and 150 mg of leaf material submerged in 25 ml of H_2O containing 0.1% biopower (Bayer Crop Science, Leverkusen, Germany) and either 50 μM of chlorotoluron (Sigma Aldrich) or 50 μM of fenoxaprop acid (Sigma Aldrich). After 24 h, leaf material (n = 5) was collected, dried, and then frozen in liquid nitrogen prior to storage at -80°C. Samples were ground in liquid nitrogen and extracted with 80% methanol (750 μl, Sigma Aldrich) overnight at 4°C, prior to centrifugation (4,500×g, 4°C, 5 min). The supernatant (5 μl) was analyzed on a Waters Xevo G2-XS QTof mass spectrometer following electrospray ionization (Waters Ltd., Wilmslow, United Kingdom) as described (Davies et al., 2020). The chlorotoluron and fenoxaprop acid metabolites were identified from their reference spectra, with metabolites quantified based on calibration curves prepared from the respective parent herbicides [chlorotoluron parent $[M–H]^+$ 213.0795, hydroxylated metabolite $[M–H]^+$ 229.0744, and fenoxaprop acid form $[M–H]^+$ 334.0482 and the respective glutathione-conjugated metabolite *S*−(6−chlorobenzoxazole−2−yl)−glutathione $[M–H]^+$ 459.0741].

Enzyme-Linked Immunosorbent Assay for AmGSTF1

Total protein from 3–5 five-leaf stage of untreated black-grass was extracted from 100 mg of leaf tissue as described by Comont et al. (2020). Total protein concentration was determined (Bio-Rad protein assay kit, Bio-Rad, Hercules, CA, United States) using bovine serum albumin (BSA; Sigma Aldrich) as a standard.

*Am*GSTF1 levels in protein samples were quantified by ELISA using specific sheep antibodies raised against *Am*GSTF1 in 96-well plates (Comont et al., 2020). Briefly, 96-well plates were coated with the primary antiserum (S909-D, diluted to 1 μg/ml in phosphate-buffered saline (PBS) for 16–18 h at 4°C. Plates were washed four times with wash buffer [PBS with 0.1% (v/v) Tween 20 (Sigma Aldrich)] before black-grass protein (100 μg) extracts were applied. After 1 h incubation, plates were washed four times, and the secondary antiserum conjugated with horseradish peroxidase (S908D-HRP diluted to 25 ng/ml) was applied and incubated for 1 h. Then, plates were washed four times, and afterward, colorimetric tetramethylbenzidine reagent (TMB; Sigma Aldrich) was applied. Reactions were terminated after 30 min with 3 M of HCl, and the absorbance at 450 nm was determined. *Am*GSTF1 protein concentration was calculated from a standard curve prepared from pure recombinant *Am*GSTF1 protein. Data were analyzed by one-way ANOVA, followed by Tukey's honestly significant difference (HSD) *post hoc* test (SPSS version 26 software, IBM, Chicago, IL, United States). The assumption of homogeneity of variance for one-way ANOVA was tested by Levene's test.

RNA Extraction and Illumina Sequencing

The aerial tissue from untreated one to two tiller black-grass plants (4 weeks) were used for RNA extraction, analyzing five biological replicates of the HS, NTSR2, and NTSR3

populations, each comprising five co-harvested plants. Total RNA was extracted from frozen tissues, using NucleoSpin RNA plant kits (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Library construction, Illumina sequencing, and *de novo* transcriptome assembly were performed by Genomics Services, Earlham Institute (Norwich, United Kingdom) as described in **Supplementary Material**. All the sequencing data were deposited in the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) (GSE162422).

RNA-Seq *de novo* Assembly and Gene Functional Annotation

De novo transcriptome assembly was carried out with Trinity v2.8.5 using default parameters (Haas et al., 2013), with the trimmomatic option added to perform quality testing and adapter trimming (MacManes, 2014). Coding regions of assembled transcripts were identified using TransDecoder v5.5.0 (Haas et al., 2013) with default parameters and the "single_best_only" option to define a single open reading frame (ORF) per transcript.

High-throughput protein function annotations for all protein sequences were obtained using the human readable description (HRD) and Gene Ontology (GO) terms, with InterPro classification and predictive domain analysis. Proteins were annotated using the AHRD (Automated Assignment of Human Readable Descriptions v.3.3.3[1]) using settings to define HRDs in favor of GO predictions. BLASTp analyses were performed against the *Arabidopsis thaliana* TAIR10 reference protein dataset[2] and the Viridiplantae protein sequences from UniProt, Swiss-Prot, and TrEMBL datasets (data downloaded on 30 January 2020). GoA mapping was obtained from UniProt[3], and InterPro annotations were retrieved from the InterPro database[4]. AHRD annotation quality was measured through three characters, namely, (1) bit score of the blast results higher than 50% with an *e*-value lower than e-10; (2) represented and overlap of the blast results higher than 60%; and (3) top token score of the assigned HRD higher than 0.5.

Analysis of Transcript Expression and Identification of Differentially Expressed Genes

The analysis of transcript expression level was performed using salmon with autodetect strandedness and validation mapping parameters. Tximport was used to transform the salmon expression count data into the DESeq dataset (Soneson et al., 2016) utilizing DESeq2 (version 1.26.0) (Love et al., 2014). The comparison was set using the HS black-grass population as the baseline/control condition. *P*-values of each comparison were then adjusted following a Benjamini–Hochberg analysis that controls the false discovery rate (FDR) (Storey and Tibshirani, 2003). Genes counted as $n < 1$ across all the samples were removed. Counts were transformed and normalized using the DESeq2 package version. Principal component analysis (PCA) was performed with all the normalized counts using the function prcomp with the center and scale options of the package stats and plotted using the fviz pca ind function of the factoextra package version in R environment.

Analysis of Herbicide Detoxification and Putative Non-target Site Resistance Biomarker Genes

Pairwise comparison of differences in the transcript expression of NTSR2 vs. HS and NTSR3 vs. HS was analyzed. Upregulated and downregulated genes were identified using the cut-off of $\log_2$FC ≥ 1 and adjusted *P*-value (FDR) ≤ 0.05, and $\log_2$FC ≤ 1 and adjusted *P*-value (FDR) ≤ 0.05, respectively. Detoxification differentially expressed genes (DEGs) including CYPs, GSTs, UGTs, and ABC transporters were identified for each comparison. ORFs of all detoxification (xenome) proteins were compared with those in the NCBI[5] database using BLASTp[6] in order to identify the xenome families.

The upregulation in xenome genes, notably members of the CYP and GST families in NTSR2 and NTSR3, were compared with that reported in the NTSR1 Peldon population derived from ion torrent next-generation sequencing (Tétard-Jones et al., 2018). In addition, the protein sequence of the different isoforms of the *Am*GTSF1 (phi family) identified by Cummins et al. (2013) were searched and identified by BLASTp within the GSTFs differentially expressed in each population. Due to the diversity of CYPs, a phylogenetic analysis was conducted to classify family and subfamily members in black-grass, as described in **Supplementary Material**. The tau (U) GSTs identified were compared with the respective genes found to be enhanced in Peldon NTSR1 plants including *Am*GSTU1, *Am*GSTU2a, *Am*GSTU2b, *Am*GSTU3, *Am*GSTU4, *Am*GSTU5, *Am*GSTU6, and *Am*GSTU7 (Nandula et al., 2019). In addition to the detoxification genes, putative NTSR biomarkers associated with the previous proteomic analyses of these populations were also analyzed (Tétard-Jones et al., 2018). These included the stem-specific protein TSJT1, 12-oxophytodienoate reductase 1 (OPR-1), D-3-phosphoglycerate dehydrogenase 1, NAD-dependent epimerase/dehydratase, and NADPH quinone oxidoreductase.

Weighted Gene Co-expression Network Analysis

Co-expression networks were constructed using the WGCNA package (version 1.69) (Langfelder and Horvath, 2008). Normalized counts were filtered in function of variance (cut-off 0.8), keeping 54,728 unigenes where abundance was then log-transformed before performing the WGCNA analysis[7]. To satisfy the approximate scale-free network distribution criterion

[1] https://github.com/groupschoof/AHRD

[2] https://www.araport.org

[3] ftp://ftp.ebi.ac.uk/pub/databases/GO/goa/UNIPROT/goa_uniprot_all.gaf.gz

[4] ftp://ftp.ebi.ac.uk/pub/databases/interpro/61.0/interpro.xml.gz

[5] https://www.ncbi.nlm.nih.gov/

[6] https://blast.ncbi.nlm.nih.gov/

[7] https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/faq.html

(Zhang and Horvath, 2005), the soft threshold power β was used to raise the co-expression similarity matrix and assess adjacency from 1 to 20 (Langfelder and Horvath, 2008) using the PickSoftThreshold function. A value of 18 was chosen to fulfill the scale-free topology criterion[8]. Afterward, the topological overlap measure (TOM) and the correspondent dissimilarity matrix (1—TOM) were calculated using the bicor correlation, which is based on an adjacency matrix, and it has been demonstrated to be more powerful and robust than Spearman correlation and Pearson pairwise analysis (Song et al., 2012). For each module of coregulated genes, a specific color was assigned to visualize the results.

Identification of Non-target Site Resistance Modules

To identify modules associated with the respective NTSR phenotypes, the eigengene modules were calculated and correlated with the herbicide resistance trait of each population using a Pearson correlation to generate a heatmap. Modules correlating with a P-value < 0.05 were selected for further characterization.

Identification of HUB Genes and Functional Annotation

To identify transcripts linked to specific NTSR traits, the respective "HUB" genes were identified within the selected modules, as unigenes having an absolute value of module membership (MM) >0.8 and a gene significance (GS), Pearson correlated with the phenotypic traits of >0.2. Common annotations of HUB unigenes between modules were then retrieved to identify potential common mechanisms linked to specific NTSR phenotypes. Venn diagrams representing the annotations of the HUB genes were then plotted using the function Venn diagram of the Venn Diagram package in R environment. To further characterize HUB genes, GO, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of the respective gene list from each selected module was performed using topGO package in R (version 2.38.1.) and ClusterProfiler package (version 3.14.3), respectively. In each case, GO enrichment was performed using Fisher's exact test with a node size of 5 and the weight01 algorithm (a mixture between the elim and weight algorithms). Genes associated with each enriched GO term were also determined. Only GO terms with P-value < 0.05 were further analyzed.

In order to perform the KEGG enrichment, the 199,761 protein sequences that formed the universe were used as query in the KAAS-KEGG Automatic Annotation Server (single-directional best hit method with the GHOSTX search) in order to assign a KO number. As the server limited the number of organisms selected as gene datasets, only 17 were selected (ath, cho, osa, dosa, ats, zma, psom, soe, bvg, lsv, han, oeu, nta, sly, vvi, gmx, and tcc), which represented 1,448,747 sequences. Pathways (ko) assigned to each KO were retrieved with the bitr_kegg function. Last, the enricher function was used for each module. Only pathways with P-value < 0.01 were analyzed.

[8]https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/R.packages/WGCNA/faq.html

Due to their known association with NTSR in black-grass, xenome genes (CYPs, GSTs, UGTs, and ABCs) and the eight molecular markers identified by Tétard-Jones et al. (2018) were also searched within the HUB genes for each key module.

Quantitative Real-Time PCR

One microgram of RNA was used for cDNA synthesis using an iScript cDNA synthesis kit for RT-qPCR (Bio-Rad, United Kingdom) in a 20 μl reaction volume. Quantitative real-time PCR was performed using a Light Cycler 96 system (Roche, United Kingdom) in a total volume of 20 μl containing 1.5 μl of cDNA prepared from 1 μg of RNA, 10 μl of Luna universal qPCR master mix (New England Biolabs, United Kingdom), and 1.2 μl of 5 μM forward and reverse gene-specific primers. The reactions were run in a three-step program including melting curve analysis and initial incubation at 95°C for 10 min, followed by amplification for 40 cycles (95°C for 10 s, 59°C for 20 s, and 72°C for 30 s) and melting curve analysis from 72 to 95°C. The specific primers of ubiquitin from black-grass (*Am*UBQ; GenBank accession number: JN599096) were used for normalization. Relative transcript expression of *Am*GSTF1 (GenBank accession number: AJ010453) was calculated based on an efficiency corrected model (Pfaffl, 2001). Mean relative transcript expression from five biological replicates ($n = 5$) was used for statistical analysis (one-way ANOVA followed by Tukey's HSD *post hoc* test, SPSS software version 26.0). Primer sequences are listed in **Supplementary Table S1**.

Genevestigator Analysis

To investigate the potential links between NTSR mechanisms and changes in gene expression associated with other plant stress responses, the sequences of unigenes from HUB gene list identified in selected modules were blasted using local BLASTp (Camacho et al., 2009) against the genome of three cereals wheat (*Triticum aestivum* IWGSC), barley (*Hordeum vulgare* IBSC_v2), and maize (*Zea mays* B73_RefGen_v4) retrieved from ftp://ftp.ensemblgenomes.org/pub/plants/release-48/fasta/). Only the BLASTp results with E-values ≤ 0.01 were used for analysis. The Genevestigator (Hruz et al., 2008) signature tool of the orthologous genes identified by BLASTp in each of the crops was then used to interrogate for similarities in gene expression changes in 2,078 experiments in wheat, 912 in barley, and 2,536 in maize mRNASeq datasets. As the Genevestigator signature tool allowed a comparison using a query of 400 genes, only the genes with higher GS were included for each module.

RESULTS

Differential Levels of Enhanced Herbicide Metabolism and AmGSTF1 in the Non-target Site Resistance Populations

To test for enhanced herbicide metabolism in the resistant black-grass plants, metabolism studies with the herbicides chlorotoluron and fenoxaprop acid were performed with all three

NTSR and the HS populations over 24 h. These herbicides were selected as they undergo different routes of metabolism, with chlorotoluron being detoxified by ring hydroxylation catalyzed by CYPs, whereas fenoxaprop acid is acted on by GSTs, to form the *S*−(6−chlorobenzoxazole−2−yl)−glutathione (CBO-SG) conjugate. For each set of plants, the levels of the two parent herbicides and their respective primary detoxification products were determined in the leaves using a high-performance liquid chromatography (HPLC)–MS method (Davies et al., 2020). With chlorotoluron, as compared with the HS population, levels of parent herbicide were depleted in both the NTSR1 and NTSR2 plants, and this was accompanied by an increased accumulation of hydroxylated chlorotoluron (**Figures 2C,D**). In the case of fenoxaprop, while more of the CBO-SG glutathione conjugate was determined in NTSR1 and NTSR2, the levels of parent fenoxaprop were equivalent to those determined in the HS plants, suggesting the herbicide was in some type of equilibrium between soluble and insoluble forms (**Figures 2A,B**). In both cases, the significance of enhanced metabolite formation was confirmed by one-way ANOVA; $P_{(CBO-SG)} = 0.02$, $P_{(hydroxylatedchlorotoluron)} = 0.04$). In contrast, the NTSR3 plants showed no difference in metabolite formation with either herbicide as compared with the HS population (**Figures 2B,D**; one-way ANOVA; $P_{(CBO-SG)} = 0.51$,

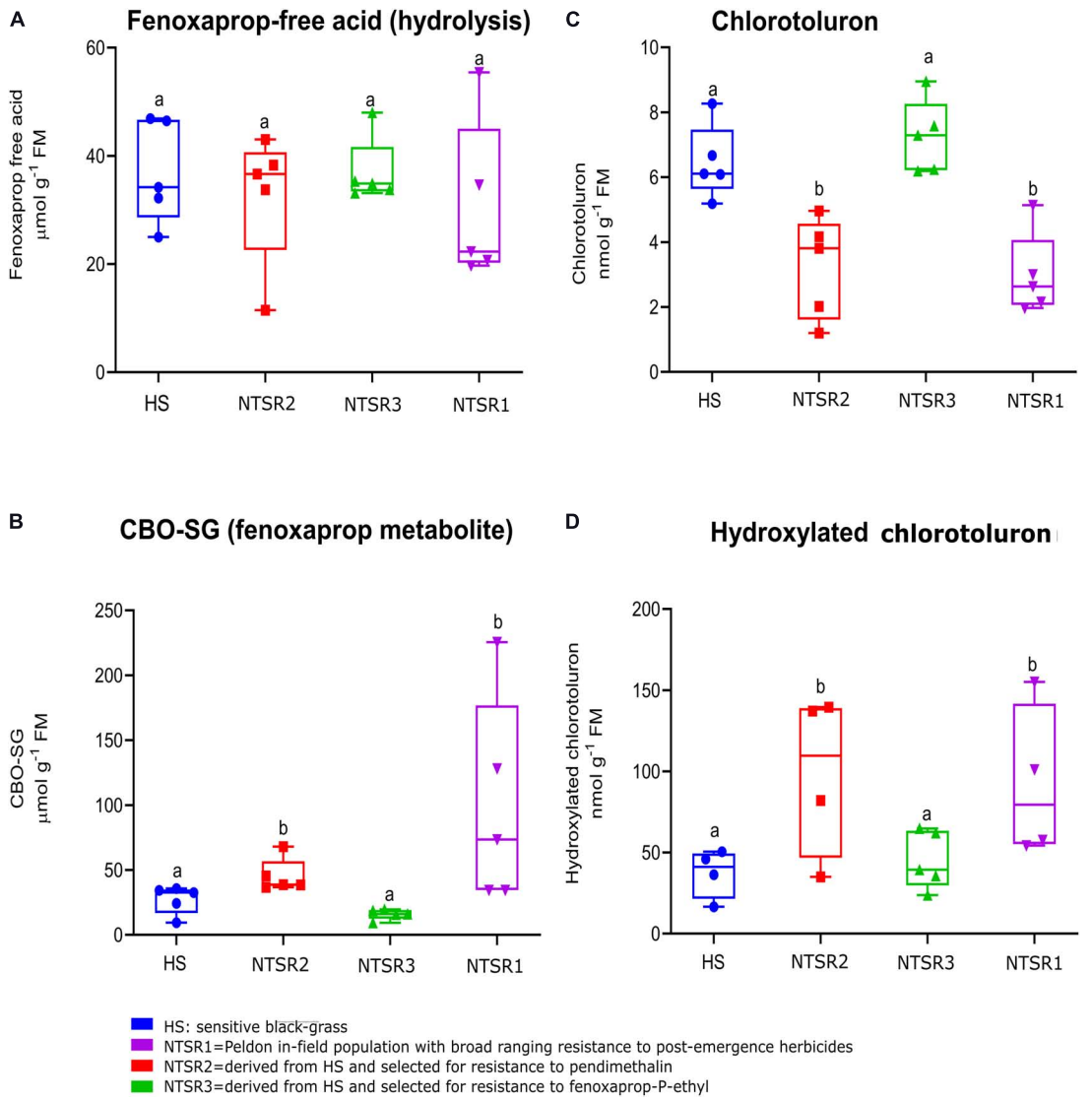

FIGURE 2 | Enhanced detoxification of fenoxaprop-ethyl and chlorotoluron in non-target site resistance (NTSR) and herbicide-sensitive (HS) populations used in this study. The average level ($n = 5$) of **(A)** fenoxaprop free acid and **(C)** chlorotoluron in leaf tissues of black-grass populations at 24 h after herbicide treatment. An average level ($n = 5$) of **(B)** fenoxaprop metabolite and **(D)** chlorotoluron metabolite at 24 h after treatment. The levels of herbicides and the main metabolites were compared among HS, NTSR1, NTSR2, and NTSR3 populations by one-way ANOVA followed by Tukey's honestly significant difference (HSD) *post hoc* test. Different letters indicate significant differences among populations ($P \leq 0.05$).

$P_{(hydroxylatedchlorotoluron)}$ = 0.65). These results indicated that like the classic NTSR1 population, the NTSR2 plants exhibited EMR, while NTSR3 did not.

As the enhanced accumulation of the biomarker *Am*GSTF1 is functionally and quantitatively linked to NTSR in black-grass populations (Cummins et al., 2013; Comont et al., 2020), the levels of this protein were determined by ELISA in each population, along with the quantitation by qPCR of the respective mRNA transcripts. As compared with the HS controls, the NTSR2 plants showed an elevated abundance of *Am*gstf1 transcripts, equivalent to that determined in the NTSR1 population (**Figure 3A**). In contrast, the *Am*gstf1 transcript expression in the NTSR3 population was not significantly different from that determined in HS plants (one-way ANOVA; P = 0.12). At the level of protein expression, all the NTSR populations contained higher concentrations of *Am*GSTF1 than the HS controls, with abundance being the greatest in the NTSR1 and NTSR2 plants (**Figure 3B**). The enhancement in *Am*GSTF1 protein, without a corresponding elevation in the respective transcripts in the NTSR3 plants, suggested that factors other than relative mRNA abundance must determine the content of this NTSR-diagnostic polypeptide in black-grass.

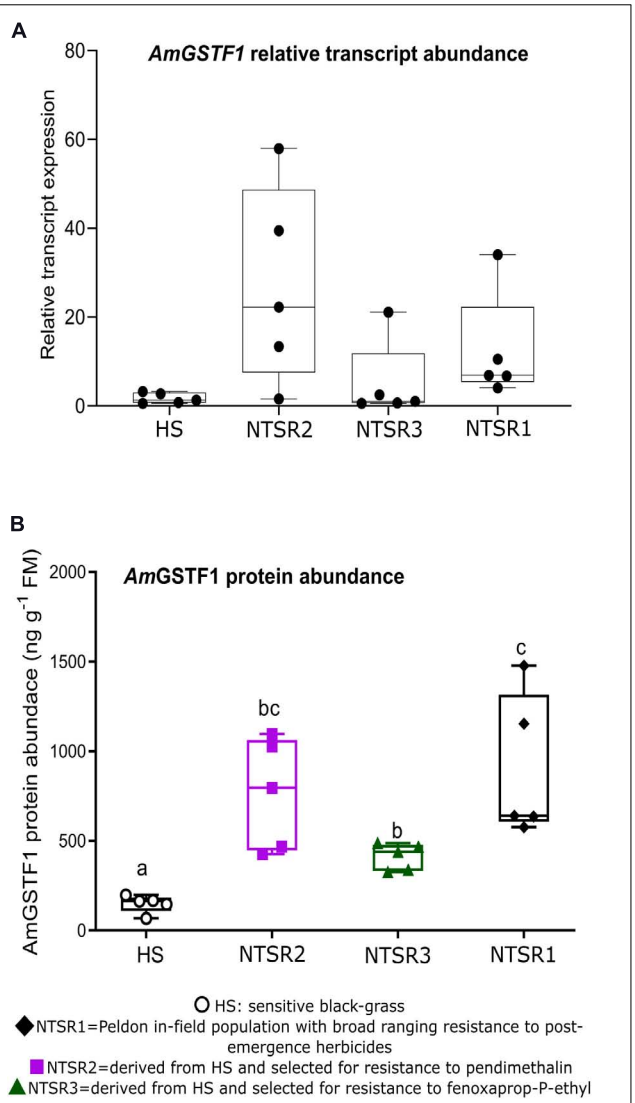

FIGURE 3 | Multiple herbicide-resistant (MHR) black-grass populations accumulate constitutively high *Am*GSTF1 protein. **(A)** Average relative transcript expression (n = 5) of *Am*gstf1 and **(B)** *Am*GSTF1 protein abundance (n = 5) in herbicide-sensitive (HS), NTSR1, NTSR2, and NTSR3 populations. The relative expression and protein levels were compared among population by one-way ANOVA followed by Tukey's honestly significant difference (HSD) *post hoc* test. Different letters indicate significant differences among populations ($P \leq 0.05$).

Illumina Sequencing and *de novo* Assembly

Having confirmed that the NTSR2 and NTSR3 populations were displaying different resistance mechanisms, the respective plants were subjected to global RNA sequencing as referenced against their parental HS plants. *De novo* assembly resulted in the identification of a total of 299,782 unigenes, originating 579,893 transcripts (**Supplementary Table S2**). The N50, which corresponds with the length of the smallest contig in the dataset necessary to represent at least 50% of the assembly (Miller et al., 2010), was 943 and 1,567 bp for unigenes and transcripts, respectively, with a guanine–cytosine (GC) content of 48.63%. In total, 199,761 coding sequences were identified from the longest read from each identified ORF, with the reads quantified using Salmon (**Supplementary Table S3**) and annotated with respect to human readable (AHRD) and GO terms.

The normalized unigenes were used to build a PCA after standardizing as a function of the NTSR2, NTSR3, and HS populations (**Figure 4**). While the PCA showed clear differences, the NTSR2 and HS populations showed several close associations. In contrast, the overall transcript expression of NTSR3 was clearly separated from that of the other two populations (principal component 2, 11.6% of the variation). The PCA further confirmed that the two NTSR populations had developed distinct transcriptomes that could not be explained by the natural genetic diversity of the populations.

Differential Expression Pattern of Xenome Genes Observed in NTSR2 and NTSR3 Populations

With their known involvement in herbicide resistance linked to enhance metabolism, it was of immediate interest to establish how the genes encoding the different families of proteins involved in xenobiotic detoxification were expressed in the NTSR2 and NTSR3 plants. As a primary analysis, the differential expression of phase 1 (CYPs), phase 2 (GSTs and UGTs), and phase 3 (ABC transporters) gene families was compared. A total of 25 CYPs, 7 GSTs, 24 UGTs, and 10 ABCs were differentially expressed in the NTSR plants relative to the HS progenitor population, with a heatmap showing the $\log_2$ fold change of each xenome gene for each population (**Figure 5**). The sequences of the respective proteins (**Supplementary Table S4**) were subject to BLASTp analysis in order to identify the family members. In total, CYPs from

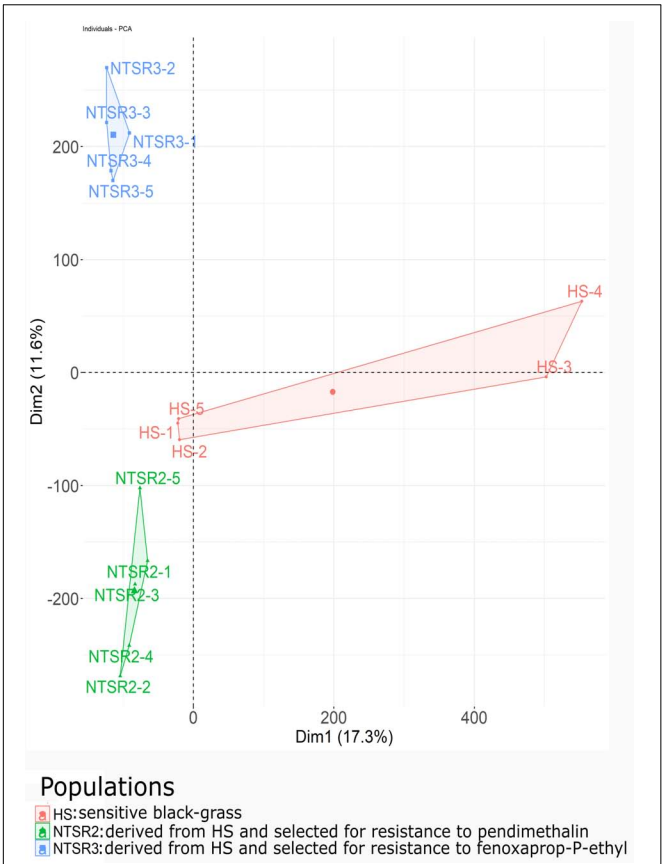

FIGURE 4 | Principal component analysis of the global transcriptional expression profiles of the NTSR2 and NTSR3 black-grass populations were distinct from each other and from HS (Rothamsted) black-grass population. Each point represents one of the five biological replicates (each containing five plants) of each population. Axes represent the percentage of variance explained by each dimension, with the dimension 1 (Dim1) explaining 16.8% and the dimension 2 (Dim2) explaining 11.6% of the variance. HS, sensitive population; NTSR2, derived from HS and selected for resistance to pendimethalin; NTSR3, derived from HS and selected for resistance to fenoxaprop-*P*-ethyl.

11 families were identified (704, 709, 71, 711, 72, 73, 76, 81, 86, 89, and 99). Relative to the HS plants, the largest number of CYP DEGs (19) was determined in the NTSR3 plants, with 8 upregulated and 11 downregulated. These DEGs belonged to all CYP families, except family 81. In contrast, only six CYPs, belonging to families 704, 709, and 81, were differentially expressed in the NTSR2 population. The CYP 81, 71, 72, and 709 families were of particular interest, due to their known connection with herbicide metabolism in crops and EMR in NTSR1 Peldon black-grass. In each case, the respective genes were assigned identities based on their phylogenies (data not shown). Based on this analysis, *Am*CYP81A4 was upregulated in both the NTSR1 and NTSR2 populations, with the orthologous (93% identical), *Am*CYP81A2 (identified in NTSR1), and *Am*CYP81A3 (identified in NTSR2) also co-enhanced. In contrast, no CYPs from these families were differentially expressed in the NTSR3 population relative to the HS population.

Seven GST DEGs were identified, with two downregulated tau (U) genes in NTSR3 (*Am*GSTF1 DN5917 and *Am*GSTU11) and five upregulated in NTSR2 (*Am*GSTF1, *Am*GSTF2, *Am*GSTU10, *Am*GSTU2, and *Am*GSTU6). In order to investigate the potential common mechanisms of detoxification between the NTSR1 and NTSR2 populations, all the GSTUs previously identified in resistant black-grass were compared (Nandula et al., 2019). *Am*GSTU6, *Am*GSTU8, and *Am*GSTU10 showed elevated expression in the NTSR2 plants. *Am*GSTU8 and *Am*GSTU10 were of particular interest, as they were both very similar (91 and 92%) to an enzyme previously termed *Am*GSTU2a, which was upregulated in the NTSR1 Peldon population and showed activity toward several herbicides (Nandula et al., 2019). In contrast, the only GSTU identified in the NTSR3 plants named *Am*GSTU11, which was an ortholog of the bronze 2 gene in maize (Marrs et al., 1995) involved in anthocyanin deposition in the vacuole, showed a lower level of expression than determined in the HS population. The previous studies in NTSR1 Peldon had also identified two phi (F) class enzymes, *Am*GSTF1 and *Am*GSTF2, as being upregulated relative to HS, with *Am*GSTF1 isoenzymes known to be encoded by multiple gene variants (Cummins et al., 2013). Similarly, the current study identified two variants termed *Am*GSTF1-DN2526 and *Am*GSTF1-DN59717. *Am*GSTF1-DN2526 was upregulated in NTSR2 but not in NTSR3, while *Am*GSTF1-DN59717 expression was depressed in both populations. Four coding sequences of the *Am*GSTF1-DN2526 were retrieved, with the sequences being shorter than the four isoforms previously identified (Cummins et al., 2013). Three of these were 99, 99, and 83%, similar, respectively, to *Am*GSTF1a and b, with the other ORF showing additional 97% similarity to *Am*GSTF1d. In addition to *Am*GSTF1, *Am*GSTF2 was also identified, though its expression was only enhanced in the NTSR2 plants. This protein was 100% similar with the *Am*GSTF2 previously identified in resistant NTSR1 black-grass (Tétard-Jones et al., 2018).

With respect to other classes of xenome genes, UGTs from the families 710C, 73B, 74F, 75E, 75K, 83A, 84C, 85A, 88B, 89B, and 90A were differentially expressed. Relative to the HS plants, a total of 13 UGTs were downregulated and three upregulated in NTSR3 (*Am*UGT73B-DN93328, *Am*UGT74F-DN1260, and *Am*UGT90A-DN118977), with 9 UGTs (*Am*UGT710C-DN18987, *Am*UGT75E-DN5345_c3, four *Am*UGT75K, *Am*UGT84C-DN5345_c1, and a *cis*-zeatin *o*-glycosyltransferase-DN762) upregulated in NTSR2. Of the 10 ABC proteins identified, 4 belonged to the family C, 1 to the D, 5 to the G, and 1 to the multidrug resistance (MDR) ABC; all were downregulated in NTSR3, while only 3 family G ABCs were downregulated in NTSR2.

Attention was then focused on the NTSR biomarkers that had been clearly identified in previous proteomic studies (Tétard-Jones et al., 2018). Of the eight biomarkers, only OPR-1 and NADPH quinone oxidoreductase 1 were determined as DEGs in NTSR2 ($\log_2$FC = 4.5) and none in NTSR3 (**Table 1**). Based on the relative enhanced expression of the proteins, only OPR1 was

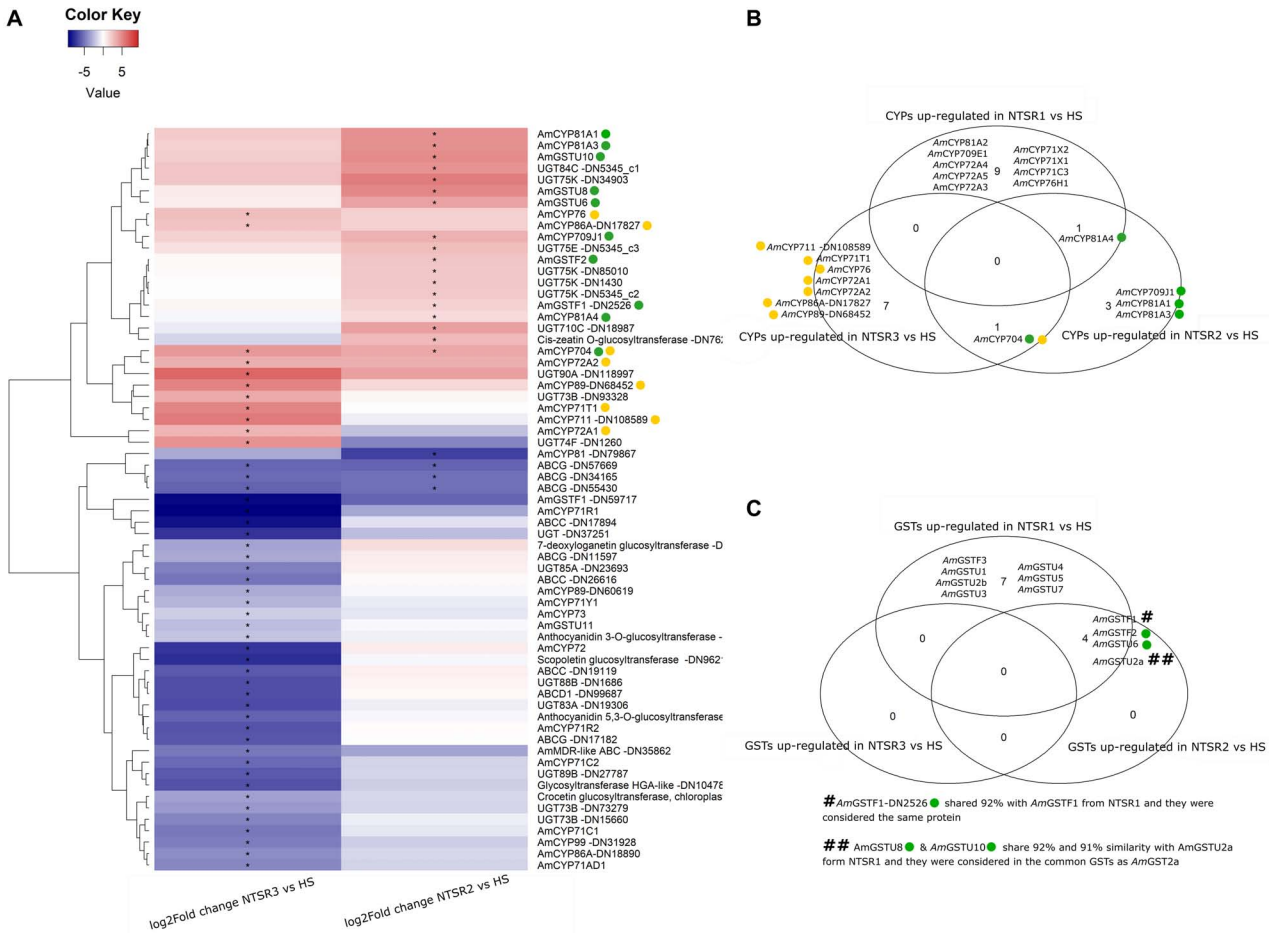

FIGURE 5 | (A) Heatmap of the $\log_2$ of the fold change of the xenome genes (including CYPs, GSTs, UGTs, and ABCs) of the NTSR2 vs. HS and NTSR3 vs. HS. Star in represent the FDR $\leq$ 0.05. Clustering dendrogram of the samples based on the $\log_2$FC was represented at the left of the heatmap. **(B)** Venn diagram representing the CYPs upregulated ($\log_2$FC $\geq$ 1 and FDR $\leq$ 0.05) in NTSR1, NTSR2, and NTSR3 vs. HS. **(C)** Venn diagram representing the GSTs upregulated ($\log_2$FC $\geq$ 1 and FDR $\leq$ 0.05) in NTSR1, NTSR2, and NTSR3 vs. HS. CYPs and GSTs upregulated in NTSR3 and NTSR2 were highlighted using yellow and green circles, respectively, in the heatmap and the Venn diagrams. HS, sensitive population; NTSR1, Peldon in-field population with broad-ranging resistance to post-emergence herbicides; NTSR2, derived from HS and selected for resistance to pendimethalin; NTSR3, derived from HS and selected for resistance to fenoxaprop-*P*-ethyl; FDR, false discovery rate; CYPs, cytochrome P450s; GSTs, glutathione transferases.

TABLE 1 | Expression of five out of the eight molecular markers identified by Tétard-Jones et al. (2018) that are not part of the xenome.

	Global expression in this study				FC of protein abundance as reported by Tétard-Jones et al. (2018)	
	$\log_2$FC NTSR2 vs. HS	FDR NTSR2 vs. HS	$\log_2$FC NTSR3 vs. HS	FDR NTSR3 vs. HS	NTSR2 vs. HS	NTSR3 vs. HS
Am 12-oxophytodienoate reductase 1 (OPR-1)	4.5	1.20E-43	−0.8	1.0	4.1	1.1
Am D-3-phosphoglycerate dehydrogenase 1	1.6	NA	−2.2	NA	3.2	1.2
Am NADPH quinone oxidoreductase 1	4.5	0.1	−2.1	1.0	1.7	1.1
Am stem-specific protein TSJT1	0.5	1.0	−0.4	1.0	1.7	1.1
Am NAD-dependent epimerase/dehydratase	0.2	1.0	0.1	1.0	1.7	1.1

The table includes the protein amount in each population in order to compare the transcriptome with the proteomic data. HS, herbicide-sensitive population; NTSR2, derived from HS and selected for resistance to pendimethalin; NTSR3, derived from HS and selected for resistance to fenoxaprop-P-ethyl; FC, fold change; FDR, false discovery rate; Am, Alopecurus myosuroides.

determined as being strongly induced in the transcriptome of the NTSR2 plants (**Table 1**).

Weighted Gene Co-expression Network Analysis of NTSR2 and NTSR3 Populations

In contrast to NTSR2, the large-scale downregulation of detoxification-related transcripts in the NTSR3 population further suggested that mechanisms other than EMR were responsible for resistance in these plants. To couple changes in transcript expression to resistance traits in the NTSR2 and NTSR3 plants, a WGCNA was performed on a total of 54,728 unigenes (variance cut-off 0.8), allowing for genes showing similar expression patterns to be grouped into modules, giving potential insight into their functional relatedness. This analysis grouped transcripts into 33 modules, each assigned a specific color, which correlated with putative resistance traits in the NTSR2 and NTSR3 populations (**Figure 6**). Three unique modules (turquoise, pink, and blue) that showed an absolute Pearson correlation coefficient (r) > 0.8, $P \leq 0.05$, were of particular relevance and were further analyzed. On the basis of positive correlation, the turquoise module had the highest module–trait relationship with the NTSR3 phenotype (r = 1; P = 3E-16), while the blue module related to the NTSR2 phenotype (r = 0.95; P = 7E-08; **Figure 6**). In contrast, the pink module showed a high negative Pearson correlation (r = -0.83 and P-value = 1E-04) with the NTSR3 phenotype.

To connect transcript expression to phenotypic traits, HUB genes were retrieved on the turquoise, pink, and blue modules. Highly connected HUB genes typically influence the expression of other genes and may be causal factors in phenotypic traits. To identify HUB genes, the correlation between the GS and the MM was plotted (**Supplementary Figures S1E–G**), with the GS representing the absolute value of the correlation between each unigene in the analysis and the phenotype, while the MM measures how connected a given gene is to other genes in the same module. The three selected modules showed a high correlation between the GS and MM, indicating that the majority of the genes in these modules are linked to traits that define each respective population (**Supplementary Figure S1**). Next,

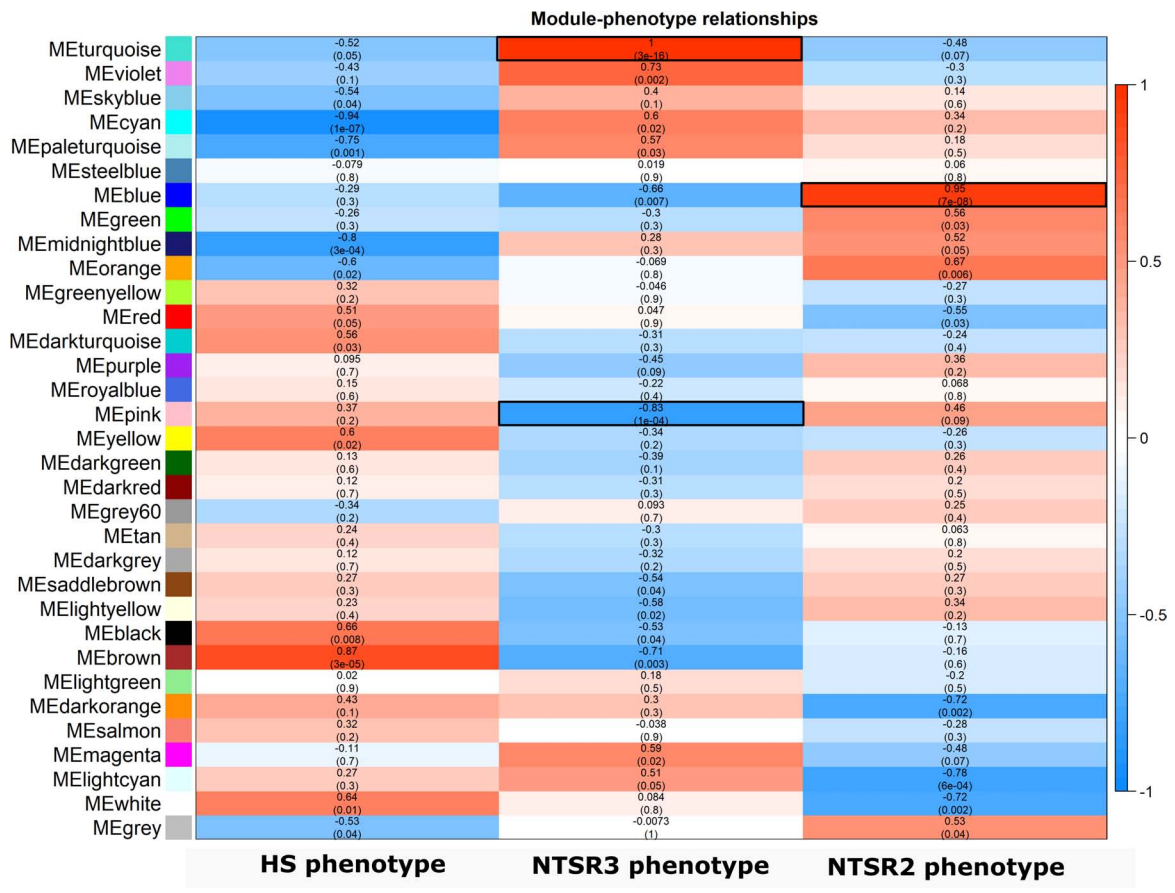

FIGURE 6 | Heatmap of the bicor correlation between the eigengenes of each WGCNA modules. Each row represents a module eigengene and each column a phenotype determined by the resistant or sensitive to the different herbicides. *R*-value and *P*-values of the bicor correlation between the eigengenes and the phenotypes are indicated in each square. In red, positive correlations; and in blue, negative correlations. HS, sensitive population; NTSR3, derived from HS and selected for resistance to fenoxaprop-*P*-ethyl; NTSR2, derived from HS and selected for resistance to pendimethalin; WGCNA, weighted gene co-expression network analysis.

the criteria of GS $\geq$ 0.2 and MM $\geq$ 0.8 applied to identify HUB genes. A total of 2,503, 263, and 1,053 HUB genes were identified in the turquoise, pink, and blue modules, respectively, with the annotations and individual GSs and MMs listed in **Supplementary Table S5**.

HUB Gene Analysis Reveals Fundamental Differences in Resistance Mechanisms in NTSR2 and NTSR3 Plants

To identify the function of genes that are important for the two NTSR traits, HUB genes were identified for each module and grouped by function using AHRD (human readable) annotation (**Supplementary Table S6**). In total, 547, 126, and 306 different annotations, respectively, were found in the HUB gene list of the turquoise, pink, and blue modules (**Supplementary Table S6**). A Venn diagram of the common and different annotations of HUB genes in each module showed that 24 different annotations were shared among the HUB genes of the three modules, including GSTs disease resistance proteins and GSTs (**Figure 7** and **Supplementary Table S6**).

While the NTSR2 and NTSR3 populations showed clearly distinct phenotypes, 73 annotations were shared between the respective turquoise and blue modules. These annotations included xenome genes, such as CYPs and glycosyltransferases, as well as transposable elements (TEs), including retrovirus-related polyproteins from transposon TNT1-94 and Ty3-gypsy retrotransposons. Other transcripts corresponding to the proteins Dicer, Argonaute, SUVH5, SUVH6, and members of the CACTA transposon En/Spm subclass were only found in the HUB list of the turquoise and pink modules exclusively associated with the NTSR3 phenotype (**Figure 7**).

Different xenome genes were identified in each module, turquoise (7 CYPs, 3 GSTs, 8 UGTs, and 4 ABCs), pink (1 GST and 1 ABC), and blue (5 CYPs, 1 GSTs, 12 UGTs, and 4 ABCs) (**Supplementary Figure S2**) and compared against

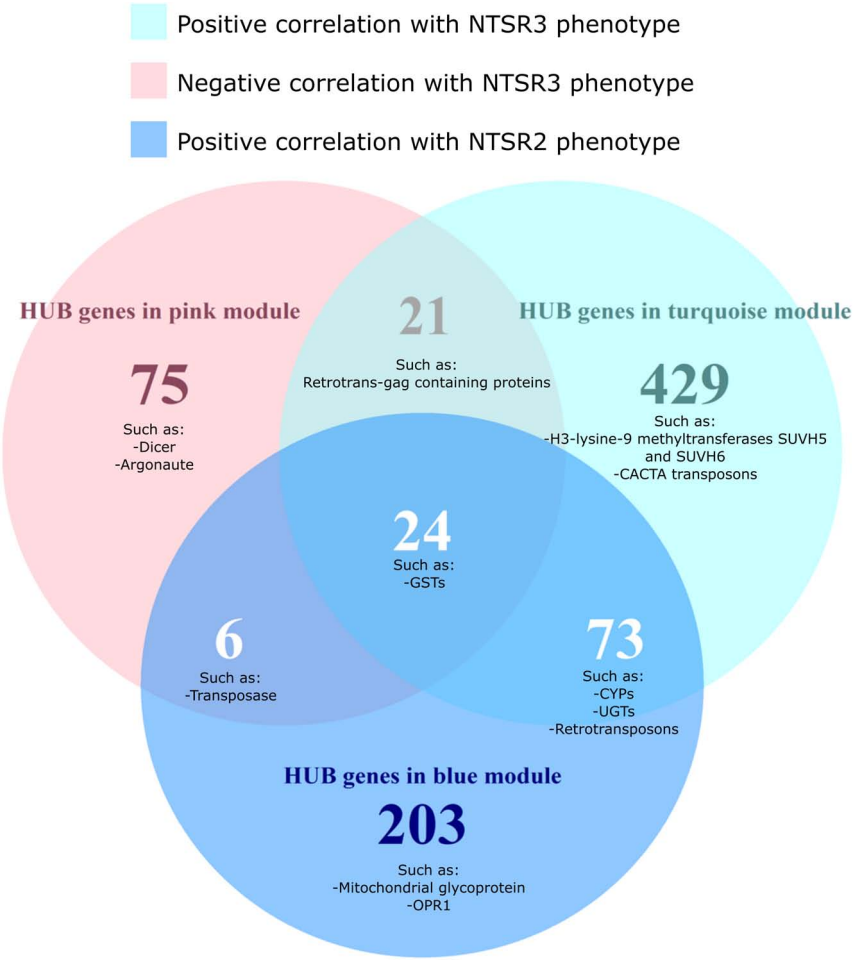

FIGURE 7 | Venn diagram of the differentially expressed HUB genes of the turquoise, pink, and blue modules grouped by the AHRD annotations. The number on each circle represents the number of different AHRD annotations. Human readable description of the common HUB genes between the turquoise (positively correlated with the NTSR3 population, pink (negatively correlated with the NTSR3 population), and blue (positively correlated the NTSR2 population) modules. HS, sensitive population; NTSR3, derived from HS and selected for resistance to fenoxaprop-*P*-ethyl; NTSR2, derived from HS and selected for resistance to pendimethalin.

the xenome genes identified by DEG analysis. Despite there being 32 CYPs and GSTs DEGs identified in NTSR3 compare with the HS population, only *Am*CYP72A1 was also identified in the HUB list of the turquoise module (**Supplementary Figure S2A**). The other 10 CYPs were not differentially expressed but, according to the WGCNA, have a role in determining the NTSR3 phenotype. Interestingly, AmGSTF_2526_c0, which was 60% similar to *Am*GSTF1 previously identified in NTSR1 Peldon, was identified as a HUB gene in the pink module, which negatively correlates with the NTSR3 phenotype. In NTSR2, out of the 11 upregulated or downregulated CYPs and GSTs, only *Am*CYP81A4 and *Am*GSTU10 were identified by both analyses (**Supplementary Figure S2B**), highlighting them as potential functional detoxification biomarkers. Within the additional biomarkers identified by Tétard-Jones et al. (2018), only OPR1 was part of the HUB gene list of the blue module correlating with the NTSR2 phenotype, being highly upregulated in this population (**Figure 7**). The other biomarker proteins were not identified on the HUB gene list, as they did not pass the cut-off defined in this study.

A GO enrichment was then performed on the HUB gene list of the three selected modules. A total of 414, 68, and 184 unigenes within the HUB genes of the turquoise, pink, and blue modules were annotated to GO terms, respectively. GOs with a *P*-value of the Fisher test ≤ 0.05 are shown (**Table 2**), with the full list of genes associated with each term presented in **Supplementary Table S7**. The greatest enrichment in GO terms in the turquoise module positively correlating with the NTSR3 phenotype was genes involved in biological process such as transposition and ADP binding (**Table 2**). The enrichment of genes such as those encoding the CACTA protein, which are involved in transposition, coincided with the identification of this type of TE in the same module (**Figure 7**). Genes with molecular functions linked to ADP binding were also enriched in the turquoise module, including disease resistance proteins and NB-ARC (nucleotide-binding adaptor shared by APAF-1, resistance proteins, and CED-4), which are required for activating plant immunity. KEGG enrichment of these modules also showed an association with the flavone and flavonol biosynthesis pathway (**Supplementary Table S8**). To further investigate the potential significance of these HUB genes, the change in their expression was compared with that determined in other plants exposed to different treatments using Genevestigator. When the HUB genes representing 16% of the total present in the turquoise module were compared against the different RNA-Seq experiments performed in wheat, barley, and maize, the 400 most significant genes were linked to induction by biotic stresses caused by infection with the fungal pathogen *Fusarium graminearum*, or with osmotic stress (**Supplementary Table S9**).

The most enriched GO terms in blue module linked to the NTSR2 phenotype were genes encoding proteins involved in calcium binding (EF-hand domain-containing protein) organelle function (mitochondrial glycoprotein) and zinc ribbon domain-containing proteins (**Table 2** and **Supplementary Table S6**). From this analysis, it was perhaps surprising that GO terms such as GO:0098754 linked to detoxification were not enriched in any module, especially in the module linked to NTSR2, even though several xenome genes were identified as HUB genes (**Supplementary Figure S2**). However, KEGG enrichment did show a link between the blue module and the caprolactam degradation pathway, which is linked to xenobiotic metabolism (**Supplementary Table S7**). Genevestigator analysis of the HUB genes from the blue module against other crop datasets showed multiple links to genes induced by biotic and abiotic stresses. In comparison with wheat, 22 genes of the blue module resembled responses to cold stress and infection by *Puccinia striiformis* (causal agent of yellow rust), while the expression of 375 genes of the same module identified an orthologous response in barley to infection by *F. graminearum*. On comparison with maize, the expression of 334 genes of the blue module resembled the response to submergence stress (**Supplementary Table S9**).

DISCUSSION

In this study, we have utilized WGCNA to search for differences in NTSR mechanisms arising in two populations of black-grass, each selected from a common ancestor by repeated treatment with different herbicides. This study did not include inducible resistance mechanisms triggered after herbicide treatment but instead focused on constitutive NTSR mechanisms (Cummins et al., 2009; Délye, 2013). As compared with HS parent plants, the NTSR2 population, selected through 8 years of consecutive treatment with the preemergence herbicide pendimethalin was shown to have an enhanced ability to detoxify chlorotoluron. As such, the NTSR2 trait showed many similarities to the EMR demonstrated in the classic NTSR1 population Peldon. Network analysis demonstrated a strongly positively correlated group of genes; the blue module linked to the NTSR2 phenotype that included CYPs and GSTs likely to function in the detoxification of pendimethalin and other selective herbicides. In contrast, in the NTSR3 plants selected for resistance to fenoxaprop-*P*-ethyl over six consecutive years, detoxification genes were not central to the resistance trait, with a distinct turquoise module of genes more functionally similar to patterns of gene expression linked to responses to pathogen and drought stress in cereals being observed.

In examining the role of xenome genes in NTSR in untreated black-grass plants, a large number of CYPs, GSTs, UGTs, and ABCs were identified as DEGs. In the NTSR2 population, relative to HS plants, multiple CYPs, GSTs, and UGTs were upregulated, while surprisingly, the ABC transporters were downregulated. In contrast, in the NTSR3 plants, the majority of xenome genes were downregulated irrespective of function. Based on their known role in the primary detoxification of herbicides, the CYP and GST superfamilies were of particular interest in the NTSR2 resistance mechanism. In wild grasses, CYPs are involved in the initial metabolism and inactivation of herbicides acting on ACCase (Kreuz and Fonné-Pfister, 1992; Ahmad-Hamdani et al., 2013), ALS (Iwakami et al., 2014a, 2019), and other modes of action (Evans et al., 2017). The data presented here point to the importance of the CYP81A family in NTSR in the NTSR2 population. *Am*CYP81A4 was enhanced in both NTSR2 and NTSR1 Peldon populations and was among the HUB genes of

TABLE 2 | Gene Ontology (GO) enrichment (biological process, molecular function, and cellular component) of the HUB gene list of the turquoise, pink, and blue WGCNA modules highly correlated with the NTSR2 and NTSR3 phenotypes.

	GO term	% significant genes	*P*-value
Turquoise module (HUB genes). Positive correlation with NTSR3 population phenotype	Protein-containing complex assembly (GO:0065003)	15.79	0.007
	Guanosine tetraphosphate metabolic process (GO:0015969)	15.38	0.038
	Transposition (GO:0032196)	14.29	0.043
	ADP binding (GO:0043531)	2.92	0.014
Pink module (HUB genes). Negative correlation with the NTSR3 population phenotype	Calmodulin binding (GO:0005516)	5.00	0.036
	Helicase activity (GO:0004386)	4.76	0.038
Blue module (HUB genes). Positive correlation with NTSR2 population phenotype	Hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds, in linear amides (GO:0016811)	33.33	0.00004
	Calcium ion binding (GO:0005509)	3.66	0.027
	Mitochondrial matrix (GO:0005759)	20.00	0.044
	SAGA-type complex (GO:0070461)	20.00	0.044
	Mitotic sister chromatid cohesion (GO:0007064)	20.00	0.014
	Organelle organization (GO:0006996)	6.67	0.018

Number of significant genes was calculated by dividing the number of significant genes in the dataset by the total of genes annotated to that GO term. WGCNA, weighted gene co-expression network analysis.

the WGCNA blue module that correlated with the respective resistance phenotype. A growing body of evidence now points to a central role for members of the CYP81A family in EMR in wild grasses (Iwakami et al., 2014a, 2019). Other CYPs upregulated in the NTSR2 plants included members of families 704 and 709, though WGCNA analysis did not point to these as being essential HUB genes.

Intriguingly, other CYP family members were identified as upregulated DEGs in the NTSR3 plants that did not evidence enhanced herbicide metabolism, including CYP711, a CYP71, and two CYP72s, as well as a CYP76, a CYP86, and a CYP89. One of these genes, *Am*CYP72A1, was identified as an NTSR3 HUB gene. Since the NTSR3 phenotype does not involve EMR, this would suggest that these induced CYPs must be playing roles in endogenous metabolism rather than in xenobiotic detoxification. Intriguingly, many related genes have also been reported to be upregulated in other NTSR weeds. These include *Ep*CYP71AK2 and *Ep*CYP72A254 in bispyribac-resistant *Echinochloa phyllopogon* (Iwakami et al., 2014b); members of families 71, 72, and 89 in *Lolium rigidum* populations resistant to diclofop and to pyroxsulam (Gaines et al., 2014; Duhoux et al., 2017); and *Ma*CYP76C1 and *Ma*CYP86B1 in *Myosoton aquaticum*-resistant plants (Liu et al., 2018). In addition, related genes are frequently induced by herbicide exposure, including families 72 and 81 induced in *Lolium* after treatment with iodosulfuron and mesosulfuron (Duhoux et al., 2017), *Ae*CYP71A4 in *Alopecurus aequalis* in response to fenoxaprop-*P*-ethyl and mesosulfuron-methyl (Zhao et al., 2017, 2019), and multiple CYP72As elevated in *Alopecurus japonicus* by fenoxaprop-*P*-ethyl (Chen et al., 2018). It therefore seems probable that a large number of CYPs enhanced by herbicides and/or NTSR in weeds relate to endogenous inducible stress responses to foreign compounds. As an example, unlike the CYP81As, the large CYP72A family is widely distributed in higher plants (Hamberger and Bak, 2013), assuming roles involved in the biotransformation of mono- and tri-terpenes (Leveau et al., 2019) and gibberellins (He et al., 2019). However, in cereals, some CYP72As are also known to be involved in herbicide metabolism, with *Os*CYP72A31 from *Oryza sativa* acting on bensulfuron-methyl (Ohno et al., 1991) and *Os*CYP72A18 catalyzing the hydroxylation of pelargonic acid (Imaishi and Matumoto, 2007).

On comparing CYP genes that were upregulated in more than one resistant population, *Am*CYP704, a member of a CYP family not previously linked to herbicide resistance in weeds was found to be upregulated in both the NTSR2 and NTSR3 plants. Previous transcriptome studies in NTSR1 Peldon identified several abundant CYPs, including *Am*CYP71X1, *Am*CYP71C3 and *Am*CYP71X2, which were not elevated in the NTSR2 plants (Tétard-Jones et al., 2018). The presence of these additional CYPs in Peldon may help explain the subtle differences in the resistance traits between the NTSR1 and NTSR2 black-grass. Significantly, several members of family 71 have known roles in herbicide detoxification, notably *Ta*CYP71C6v1 from wheat, which is able to metabolize sulfonylurea herbicides when expressed in yeast (Xiang et al., 2006).

GSTs, particularly *Am*GSTF1, have also been shown to have critical roles in NTSR in black-grass and other wild grasses (Cummins et al., 2013). In black-grass, *Am*GSTF1 is encoded by four genes, termed *Am*GSTF1a, b, c, and d, though only the 2c and 2d isoforms were detected in the previous proteome analysis of NTSR plants (Tétard-Jones et al., 2018). From the transcriptome data in the current study, two *Am*GSTF1 variants were identified, though the transcripts were too short

to accurately determine their identities. The ORF of *Am*GSTF-DN2526 was the most similar to the 2a or 2b isoforms. Transcripts encoding this variant were highly upregulated in the NTSR2 but not in NTSR3 plants. Given the apparently critical role of *Am*GSTF1 in NTSR in black-grass, this complex regulation of its isoenzymes is intriguing, though to date no functional significance has been attributed to the respective protein variants. Certainly, the results of the transcriptomic study reported here suggest that the transcriptional regulation of *Am*GSTF1 cannot account for its quantitative regulatory role recently confirmed in NTSR (Comont et al., 2020). While *Am*GSTF1 protein expression was elevated in both the NTSR2 and NTSR3 populations, enhanced levels of the respective transcripts were only determined in the NTSR2 plants. In addition, HUB analysis identified a sequence showing only 60% similarity to the *Am*GSTF1 transcript in the pink module, and it was therefore questionable whether *Am*GSTF1 mRNAs play a regulatory role in NTSR. Instead, out data suggested that the known regulatory function of the *Am*GSTF1 protein in NTSR must be regulated transcriptionally or post-transcriptionally.

In addition to *Am*GSTF1, the additional phi class *Am*GSTF2, which shares 100% similarity with the one previously identified by Tétard-Jones et al. (2018), was also highly upregulated in the NTSR2 but not in NTSR3 plants. As previously reported by Nandula et al. (2019), this *Am*GSTF2 shared high a similarity with other GSTFs induced by safeners in cereals, including *Zm*GSTF4 in maize and *Ta*GSTF3 in wheat, which are able to both metabolize chloracetanilide herbicides and act as glutathione peroxidases (Cummins et al., 2003).

In total, four GSTUs were differentially expressed in NTSR black-grass, with three of them being upregulated in NTSR2. Of these, *Am*GSTU10 was identified as a HUB gene in the associated blue module, further confirming its role in resistance (Tétard-Jones et al., 2018). *Am*GSTU10 shared a high similarity with the enzyme termed *Am*GSTU2a previously identified in NTSR1 Peldon, which was active toward the herbicides fenoxaprop-*P*-ethyl and metolachlor (Nandula et al., 2019). It would seem most likely that *Am*GSTU10 and *Am*GSTU2a are derived from gene duplication. Concerning other NTSR mechanisms linked to the xenome, proteomic studies had identified OPR1 as a resistance biomarker (Tétard-Jones et al., 2018). In the current study, OPR1 was also identified as a HUB gene in the blue module correlated with the NTSR2 resistance phenotype, consistent with the importance of this gene in resistance.

In our previous transcriptome and proteome studies with the Peldon NTSR1 population, several similarities were determined between NTSR in black-grass and MDR in humans (Cummins et al., 2013; Tétard-Jones et al., 2018). The current study further confirmed a link between MDR mechanisms and changes in gene expression associated with NTSR2, which most closely resembles NTSR1. Intriguingly, genes enriched in the blue NTSR2 module, such as zinc ribbon domain-containing proteins, EF-hand and calcium ion binding proteins, and *P*-glycoproteins, are also associated with MDR in cancer cells (Hong et al., 2005; Shen et al., 2012). Of relevance to xenobiotic detoxification, *P*-glycoproteins are known to be involved in the membrane transport and export of cytotoxic drugs, being associated with regulatory zinc ribbon domain-containing proteins (Hong et al., 2005; Sulová et al., 2009).

Increased expression of transcripts involved in stress responses, e.g., oxidative stress, has previously been identified in NTSR1 Peldon plants (Tétard-Jones et al., 2018). Genevestigator analysis revealed that HUB genes associated with both the NTSR2 and NTSR3 phenotypes corresponded with transcripts linked to responses to biotic (infection with *Fusarium graminearum* or *Puccinia striiformis*) and abiotic (cold and osmotic) stresses in cereals. Together, these results suggest that plant responses to adverse environmental conditions are integrally linked to NTSR-based herbicide resistance. In support of this hypothesis, a link between TEs linked to stress tolerance and NTSR was determined. TEs were enriched in the modules (turquoise and pink) highly correlated with the NTSR3 population, notably CACTA TEs and retrotransposon gag proteins (Kunze et al., 1997; Todorovska, 2007). TEs were also found in the NTSR2 population, including the long terminal repeat (LTR) retrotransposon and the *Pseudoviridae* Ty1-copia retrotransposon (Kumar and Bennetzen, 1999). TEs are a catalyst for genetic variation and are known to be activated by environmental stress (Takeda et al., 1999; Kimura et al., 2001; Scheid et al., 2010; Woodrow et al., 2011), playing critical roles in the adaptation of plants to abiotic stress (Naito et al., 2009; Scheid et al., 2010; Sigman and Slotkin, 2015). Therefore, we speculate that TEs could play a key role in the evolution of NTSR in the black-grass populations, with each phenotype associated with the recruitment of distinct groups of TEs.

In conclusion, WGCNA is a useful tool to identify important and previously unknown gene networks mediating NTSR using transcriptome datasets. In particular, the potential connection between NTSR and plant responses to (a)biotic stress mechanisms has been identified in this study. This current finding starts to shed light on the complexity of NTSR and to provide important basic information to interrogate the evolutionary route of NTSR in black-grass and other grass weeds. In addition, it will now be of interest to study the changes in the transcriptomes of these NTSR populations that result from herbicide treatment to determine chemically inducible as well as latent resistance mechanisms in black-grass.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: GEO repository (GSE162422).

AUTHOR CONTRIBUTIONS

RE conceived the study and was in charge of funding acquisition and supervision. NO cultivated and provided the plant material and performed the metabolism analysis and the ELISA. MB-H helped in the design, data curation, and revision. AW helped in the cultivation of the plants and the extraction of the RNA. AG-C helped in the extraction of the RNA and its quality control,

identified the DEGs, and performed the phylogeny trees that determined the nomenclature of the CYPs. SF-O analyzed the RNA-seq, conducted the gene correlation network, the GO and KEGG enrichment, and the Genevestigator analysis and drafted the manuscript. All authors participated in the revisions and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.636652/full#supplementary-material

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Mechanism of Resistance to *S*-metolachlor in Palmer amaranth

Gulab Rangani[1], *Matheus Noguera*[1], *Reiofeli Salas-Perez*[1†], *Lariza Benedetti*[2] *and Nilda Roma-Burgos*[1*]

[1] *Department of Crop, Soil and Environmental Sciences, University of Arkansas, Fayetteville, AR, United States,* [2] *Crop Protection Graduate Program (Programa de Pós-Graduação em Fitossanidade), Federal University of Pelotas (Universidade Federal de Pelotas), Pelotas, Brazil*

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***Correspondence:**
Nilda Roma-Burgos
nburgos@uark.edu

†Present address:
Reiofeli Salas-Perez,
Dole Philippines, Inc., Polomolok, Philippines

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Herbicides are major tools for effective weed management. The evolution of resistance to herbicides in weedy species, especially contributed by non-target-site-based resistance (NTSR) is a worrisome issue in crop production globally. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) is one of the extremely difficult weeds in southern US crop production. In this study, we present the level and molecular basis of resistance to the chloroacetamide herbicide, *S*-metolachlor, in six field-evolved *A. palmeri* populations that had survivors at the recommended field-dose (1.1 kg ai ha^{-1}). These samples were collected in 2014 and 2015. The level of resistance was determined in dose-response assays. The effective dose for 50% control (ED_{50}) of the susceptible population was 27 g ai ha^{-1}, whereas the ED_{50} of the resistant populations ranged from 88 to 785 g ai ha^{-1}. Therefore, *A. palmeri* resistance to *S*-metolachlor evolved in Arkansas as early as 2014. Metabolic-inhibitor and molecular assays indicated NTSR in these populations, mainly driven by GSTs. To understand the mechanism of resistance, selected candidate genes were analyzed in leaves and roots of survivors (with 1× *S*-metolachlor). Expression analysis of the candidate genes showed that the primary site of *S*-metolachlor detoxification in *A. palmeri* is in the roots. Two *GST* genes, *ApGSTU19* and *ApGSTF8* were constitutively highly expressed in roots of all plants across all resistant populations tested. The expression of both *GST*s increased further in survivors after treatment with *S*-metolachlor. The induction level of *ApGSTF2* and *ApGSTF2like* by *S*-metolachlor differed among resistant populations. Overall, higher expression of *ApGSTU19*, *ApGSTF8*, *ApGSTF2,* and *ApGSTF2like*, which would lead to higher GST activity in roots, was strongly associated with the resistant phenotype. Phylogenetic relationship and analysis of substrate binding site of candidate genes suggested functional similarities with known metolachlor-detoxifying GSTs, effecting metabolic resistance to *S*-metolachlor in *A. palmeri*. Resistance is achieved by elevated baseline expression of these genes and further induction by *S*-metolachlor in resistant plants.

Keywords: Palmer amaranth, S-metolachlor, tolerance, resistance, GST, gene expression, NTSR

INTRODUCTION

The evolution of resistance in response to intensive herbicide selection pressure in weedy species is a worldwide problem in crop production. Understanding the underlying physiological and molecular basis of resistance evolution is useful in long-term resistance management and design of new agrichemicals. *S*-metolachlor is a member of the chloroacetamide chemical family under Group 15 of the Weed Science Society of America (WSSA) classification system. Group 15 herbicides are soil-active and inhibit seedling root and shoot growth by blocking the formation of very long chain fatty acids (VLCFA, site-of-action), acting even before the susceptible grass or broadleaf weeds emerge. *S*-metolachlor is typically used preemergence in corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), soybean [*Glycine max* (L.) Merr] and many other crops, including turfgrass, for the control of grasses and small-seeded broadleaf weeds. Among the primary weeds controlled by *S*-metolachlor are *Amaranthus* species. In the USA, *S*-metolachlor is the third largest volume of herbicide active ingredient used, following glyphosate and atrazine (Atwood and Paisley-Jones, 2017).

Target-site and non-target-site-based resistance (NTSR) mechanisms are the evolved physiological, molecular or genetic changes in weed populations that allow them to survive, or escape, herbicide application (Jugulam and Shyam, 2019; Gaines et al., 2020). Target-site resistance is endowed mostly by modification or amplification of the target enzyme, involves single gene, and does not contribute to multiple resistance with other herbicide sites of action (SOA). NTSR includes reduced translocation, sequestration and/or metabolic degradation of herbicide to non-toxic metabolite(s), among other plant modifications. The most adverse effect of metabolism-based resistance is multiple resistance to herbicides with different SOAs, or resistance to new herbicide chemistries yet "unseen" by weeds. This is a serious threat to weed management as NTSR can eliminate the utility of many herbicide tools, which threatens global food security. Metabolic degradation of herbicides is complex. It is generally facilitated by inherited or elicited molecular responses, which involve multiple superfamily genes such as cytochrome (P450) monooxygenase and glutathione-S-transferase (GST). A similar type of enhanced molecular response is also elicited using safeners, formerly referred to as herbicide antidotes, that can increase the detoxification rates of herbicides in cereal plants (Riechers et al., 2010). Safeners are used to increase crop tolerance to certain herbicide chemistries such as chloroacetanilides (Davies and Caseley, 1999). Safeners reduce the phytotoxicity of chloroacetamides to corn, sorghum, wheat, rice and barley by inducing specific GSTs, which are the main detoxifying agents for chloroacetamides and are involved in metabolic detoxification of certain other herbicides (Cummins et al., 1997; Dixon et al., 1997; Gronwald and Plaisance, 1998; Pascal and Scalla, 1999; Wu et al., 1999; Deng and Hatzios, 2002a,b; Scalla and Roulet, 2002). Although the use of safeners is based on selective action on cereal crops (Zhang and Riechers, 2004), apparent conservation in molecular response involving safener recognition and GST induction in Arabidopsis has been observed (DeRidder et al., 2002; Edwards et al., 2005; DeRidder and Goldsbrough, 2006; Riechers et al., 2010).

Palmer amaranth *(Amaranthus. palmeri* S. Wats) is now one of the major, and most difficult, weeds to control in corn, cotton, and soybean production. Resistance to glyphosate and acetolactate synthase (ALS) inhibitors among *A. palmeri* populations is rampant. *A. palmeri* has evolved resistance to eight herbicide SOAs including that of protoporphyrinogen oxidase (PPO)-inhibitors (Noguera et al., 2020) and *S*-metolachlor (Brabham et al., 2019) in the mid-southern US, starting in Arkansas. The increase in resistance to PPO inhibitors forced farmers to rely more on VLCFA inhibitors for *A. palmeri* control, further reducing the diversity of herbicides and spectrum of control. Pre-existing NTSR to ALS- or PPO-inhibitors could also have increased the likelihood of resistance evolution to VLCFA inhibitors. In any case, this latest scenario is highly worrisome because the PPO inhibitors and VLCFA inhibitors, such as *S*-metolachlor, are the remaining pillars of chemical weed management for *A. palmeri* in various crops.

Target-site modification is an unlikely mechanism for tolerance or resistance to VLCFA inhibitors in crops and weedy species due to the multiple SOAs of different enzymes involved in VLCFA synthesis (Busi, 2014). Crop selectivity to several chloroacetamide herbicides and safeners is mediated by enhanced GST activity, as a result of increased *GST* expression (Leavitt and Penner, 1979; Lamoureux and Rusness, 1989; Frova, 2006; Riechers et al., 2010). Enhanced amount of GSTF1 protein, a biomarker of NTST was found in *L. rigidum* population that showed reduced sensitivity to VLCFA inhibitors Torra et al. (2021). Thus far, resistance to VLCFA inhibitors in weedy species is attributed to NTSR mechanism mediated by enhanced GST activity (Busi et al., 2018; Brabham et al., 2019; Dücker et al., 2020). GSTs from the phi (GSTF) and tau (GSTU) classes are unique to plants and its role has been widely investigated in stress tolerance and secondary metabolism as well as in detoxification of herbicides in crops and weeds (Hatton et al., 1996; Cummins et al., 2011). GSTs catalyze the conjugation of glutathione (GSH) with a wide Hatton et al., range of endogenous and xenobiotic molecules and protect against oxidative damage. GSTFs and GSTUs show specificity toward different substrates. Phi enzymes are highly reactive toward chloroacetanilide and thiocarbamate herbicides. Some Phi GSTs have other functions including transport of flavonoid pigments to the vacuole, shoot regeneration and GSH peroxidase activity. Tau enzymes are highly efficient in detoxifying diphenylether and aryloxyphenoxypropionate herbicides. In addition, Tau GSTs have important roles in intracellular signaling, vacuolar deposition of anthocyanin, responses to soil stresses, auxin and cytokinin hormones (Edwards et al., 2005).

In this study we determined the *S*-metolachlor resistance level in six *A. palmeri* populations, examined the expression profile of candidate *GST* genes in these resistant populations in response to *S*-metolachlor, and provided evidence for its association with herbicide detoxification.

MATERIALS AND METHODS

Plant Material

A late-season collection of *A. palmeri* inflorescences was done in the 2014–2016 summer(s) following established methodology

(Burgos et al., 2013). Six populations from four Arkansas counties were included in this study, which will hereby be identified as: 15CRI-A, 14CRI-C, 14CRI-G, 14MIS-E, 14MIS-H, and 16WOO-A. A susceptible standard (SS) collected from Crawford, AR, was also included.

Dose Response of *Amaranthus palmeri* Populations to *S*-metolachlor

To determine the resistance level to *S*-metolachlor, a dose-response experiment was conducted in the greenhouse. Fifty seeds of each population were sown in 400-mL pots filled with a 4:1 mixture of field soil:commercial potting medium and sprayed with the herbicide. Soil (Roxana silt loam) was collected at the Vegetable Research Station in Kibler, AR and mixed with Sunshine® Premix #1 (Sun Gro Horticulture, Bellevue, WA). The final physico-chemical properties of the mixture were: pH = 6.4, organic matter = 1.79%, and clay content = 15.1%. The experiment was conducted in a completely randomized design with three replications, with pot as experimental unit. The seeds were spread on the soil surface, then covered with a thin layer of the same soil mixture, and sprayed with eight rates of *S*-metolachlor (Dual II Magnum; Syngenta Crop Protection, LLC, Greensboro, NC) as follows: 0, 1/16x, 1/8x, 1/4x, 1/2x, 1x, 2x, and 3x, where x is the labeled dose for a silt loam soil (1.12 kg ai ha^{-1}). For the SS, the lowest dose was 1/32x and the highest was 2x. Herbicide application was done in a spray chamber equipped with a motorized boom fitted with flat-fan 1100067 nozzles (Teejet, Wheaton, IL), calibrated to deliver 187 L ha^{-1} at 276 kPa. After application, the herbicide was activated by gently misting water on the soil surface. The pots were then placed in a greenhouse kept at 32/28C day/night temperature and 16-h day length. Throughout the study, soil in the pots were watered by capillarity as needed. At 28 days after application (DAT), seedlings were counted and survival (%) was calculated relative to the non-treated checks. Regression analysis was done with the packages *drc* and *mselect* in R 4.0.3 (Ritz et al., 2015). The appropriate model was selected based on the Akaike's information criterion and *p*-value for the lack-of-fit test (Ritz, 2010). Data from the accessions CRI-G and MIS-E were fitted with a three-parameter Log-logistic model (Eq. 1, with $c = 0$); WOO-A, CRI-A and SS, with a three-parameter Weibull II model (Eq. 3, with $c = 0$); CRI-C with a four-parameter Weibull II model (Eq. 3); and MIS-H with a three-parameter Weibull I model (Eq. 2).

$$Y = c + \frac{d - c}{1 + \exp\left(b(\log(x) - \log(e))\right)} \quad (1)$$

$$Y = d\left(\exp\left(-\exp\left(b(\log(x) - \log(e))\right)\right)\right) \quad (2)$$

$$Y = c + (d - c)\left(1 - \exp\left(-\exp\left(b(\log(x) - \log(e))\right)\right)\right) \quad (3)$$

In the equations above, Y is the survival percentage, d is the asymptote at the upper limit, c is the asymptote at the lower limit, X is the *S*-metolachlor rate and b is the slope around e, which is the value of X that causes a 50% reduction of Y. The ED50 of each accession was estimated and used for the determination of resistance level (ED50 R/ED50 SS).

Herbicide Metabolism Inhibition by NBD-Cl

To verify the contribution of GSTs toward *S*-metolachlor resistance in *A. palmeri*, three populations (14CRI-G, 15CRI-A and SS) were tested in an agar-based plate assay with a GST inhibitor. The growth medium was prepared by dissolving 2.2 g of Murashige and Skoog basal salt mix (PhytoTech Labs Inc., Lenexa, KS) and 4 g of agar (Himedia Labs, West Chester, PA) in 500 mL deionized water. The pH was adjusted to 6.3 and the mixture was autoclaved. Analytical grade *S*-metolachlor (Sigma-Aldrich Inc., St. Louis, MO) and the GST inhibitor 4-chloro-7-nitrobenzofurazan (NBD-Cl; Sigma-Aldrich) were dissolved and diluted in chloroform. Work solutions were prepared through serial dilutions to achieve *S*-metolachlor concentrations of 0, 0.06, 0.12, 0.25, 0.5, 1, 2, 4, and 8 μM, and NBD-Cl concentrations of 0 and 25 μM in the growth medium. Herbicide and inhibitor rates were chosen based on preliminary experiments (data not shown). A final chloroform concentration of 0.14% was kept constant in all plates by adding 24 μL of each work solution to 30 mL of growth medium to each plate. The plates were sterile, square, gridded Petri dishes (100 × 100 × 15 mm, 13-mm grids, Simport Scientific Inc., Beloeil, QB, Canada). Each plate had 30 seeds. The plates were sealed with Parafilm tape (Bemis Company Inc, Neenah, WI) and kept in a growth chamber at 30/28°C day/night temperature, and 16-h daylight. At 14 DAT, the plates were photographed and root lengths were measured using ImageJ (Schneider et al., 2012). The square grids were used as scale for unit conversion. From each plate, 10–15 representative roots were measured, which were considered biological replicates. The experiment was repeated and data from both runs were pooled as results across runs did not differ statistically. Root length data were fitted to a four-parameter, log logistic model using the package *drc* in R 4.0.3, as defined previously in Eq. 1. To determine the sole effect of NBD-Cl in the absence of herbicide, a subset of the data was submitted to ANOVA and means were compared using a Tukey's HSD test in the *multcomp* package in R.

Selection of Candidate Genes

Homologs of known *GST* genes in *A. palmeri* were identified using BLAST tool from CoGe (https://genomevolution.org/coge/SearchResults.pl?s=amaranthus&p=genome). The top similar genes were identified as candidate genes. Additionally, NCBI BLAST tool was also used to examine the homology between all selected genes within selected species.

Gene Expression Analysis

For candidate gene expression analysis, survivors of 1x field rate from the resistant (R) populations were sampled. Gene expression analysis was conducted using leaf and root tissues. Three biological replicates were used for leaf tissue analysis. Three to five leaf segments, ∼0.5 cm long, were sampled from a single plant and 2–5 plants were pooled together from the same population. The leaf tissues from treated plants were harvested 21 days after *S*-metolachlor treatment. Two biological replicates of treated leaf tissues were composed of pooled tissues from at least 6–10 R plants per population and the third biological replicate was comprised of tissue collected from a single plant

from each population. Leaves of non-treated control plants from R and S populations were sampled at 21 days after planting and all three biological replicates consisted of pooled plants. Roots were collected using the same pooled plants used for leaf tissue sampling. Equal amounts of root tissue from each treated plant (resistant survivor) were pooled to represent two biological replicates per population. Root tissue collected from a single plant was not enough to conduct the expression analysis of all genes; therefore, the third biological replicate, which is a single plant sampled for leaf tissue, could not be included in the root tissue analysis. Similarly, root tissues of non-treated plants were pooled representing three biological replicates from each population. Total RNA was isolated using an E.Z.N.A ® Plant RNA isolation kit (Omega Bio-tek, Norcross, GA) and converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions using 1 μg of total RNA for each sample. The differences in transcript abundance of five candidate genes; *ApGSTU18*, *ApGSTU19*, *ApGSTF8*, *ApGSTF2,* and *ApGSTF2like* were validated by RT-qPCR using iCycler Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Each qPCR reaction contained 1X IQ^{TM} SYBR Green Supermix (2x) (Bio-Rad Laboratories), 1 μl of cDNA (1:5 dilution), and 0.5 μM of gene-specific primers. Relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ algorithm (Livak and Schmittgen, 2001) by normalizing to the expression of *ß-tubulin* (Nakka et al., 2017) and *elongation factor1α* gene. Fold change in each resistant population was measured against non-treated S plants.

Phylogenetic Analysis and Active-Site Comparison

A phylogenetic tree of GSTs from *A. palmeri*, *Zea mays*, *Arabidopsis thaliana*, *Lolium rigidum,* and *Alopecurus myosuroides* was constructed using standard configuration in MEGA-X. The H-site was added manually using NCBI CD-search tool in the candidate GST enzyme sequence. The full-length sequence of *ApGSTU19* was obtained using the primers listed in **Supplementary Table 1**. Total RNA of the two survivors from 14CRI-G to 15CRI-A population was converted to cDNA as described in the previous section. The PCR was conducted in a 20-μL volume that consisted of 1 μL cDNA, 0.5 μM of each primer, and 10 μL EmeraldAmp MAX PCR Master Mix (Takara). The PCR was run with the following profile: 98°C for 30 s; 30 cycles of 98°C for 30 s, 58°C for 30 s and 72°C for 1 min; followed by a final extension step of 10 min at 72°C. The PCR product was purified from agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and sequenced by Psomagen Inc. (Rockville, MD). The resulting overlapping fragments were assembled into one sequence to get the complete coding region of *ApGSTU19* using Sequencher (Gene Codes Corporation, Ann Arbor, MI) software. The nucleotide sequences were translated into open reading frames using ExPASy's online translation tool, and compared to the reference using Uniprot Align tool.

RESULTS

S-metolachlor Resistance Level in Field-Evolved Populations

In the general screening of 115 samples collected between 2014 and 2016 with 1x dose (1.12 kg ai ha^{-1}) *S*-metolachlor, six populations showed reduced sensitivity to *S*-metolachlor, while the rest was controlled 100% (data not shown). The ED_{50} of susceptible population (SS) was 27 g ai ha^{-1}, whereas the ED_{50} of the resistant populations ranged from 88 to 784 g ai ha^{-1} (**Figure 1**, **Table 1**). Thus, the levels of resistance to *S*-metolachlor in 16WOO-A, 15CRI-A, 14CRI-G, 14-MIS-H, 14CRI-C, and 14MIS-E ranged from 3- to 29-fold relative to the SS population (**Table 1**). Several resistant populations had survivors at the 2x dose and notably more survivors at the sublethal dose (0.5x) compared to the S population (**Supplementary Figure 1**).

Effect of GST Inhibitor and S-metolachlor on the Seedling Growth of *A. palmeri*

To verify that GST enzymes were involved in resistance to *S*-metolachlor, we tested the effect of *S*-metolachlor on seedling growth of *A. palmeri* in the presence and absence of GST inhibitor, NBD-Cl. Seeds of two resistant populations, 15CRI-A and 14CRI-G, and SS were first germinated in a medium containing a range of NBD-Cl concentrations (25 μM to 0.1 mM) to determine the maximum tolerated dose that would cause minimum injury (Data not shown). Symptoms of NBD-Cl phytotoxicity appeared in the form of stunted root and cotyledon growth even at the lowest concentration tested. The lowest concentration (25 μM) was used to study the inhibitor effect on *S*-metolachlor phytotoxicity.

Sensitivity to *S*-metolachlor was observed as inhibition of root growth in agar-based assays (**Figures 2**, **3**). Without NBD-Cl, the R and SS populations responded differently to *S*-metolachlor. The GR_{50} for SS, 15CRI-A, and 14CRI-G were 0.23, 0.92, and 0.74 μM, respectively (**Figure 2**). Based on the GR_{50}, 15CRI-A, and 14CRI-G showed resistance levels of 4.1- and 3.6-fold in relation to SS (**Table 2**) in the agar medium. With NBD-Cl, the root growth of all populations was severely reduced, with further reduction as herbicide concentration increased. However, none of the regression models tested fit the data because of the small difference between the lower and upper asymptote. This was due to the significant toxicity of NBD-Cl alone. Regardless of the high growth inhibition by NBD-Cl alone, the data portrayed the combined effect of NBD-Cl and *S*-metolachlor on resistant populations (**Figure 3**). Without NDB-Cl, the highest dose tolerated by R populations was 0.5 μM *S*-metolachlor (**Figure 3A**). Above this dose, *S*-metolachlor caused severe inhibition of root growth. Adding NBD-Cl to 0.5 μM *S*-metolachlor resulted in greater root growth inhibition compared to NBD-Cl alone. This suggests that NBD-Cl suppressed the GSTs responsible for herbicide detoxification, making the R populations less resistant to *S*-metolachlor.

The highest dose tolerated by SS population was 0.12 μM (**Figure 3B**). The SS population response to 0.12 μM herbicide did not change in the presence of NBD-Cl; instead, lower concentration of *S*-metolachlor stimulated root growth. Also,

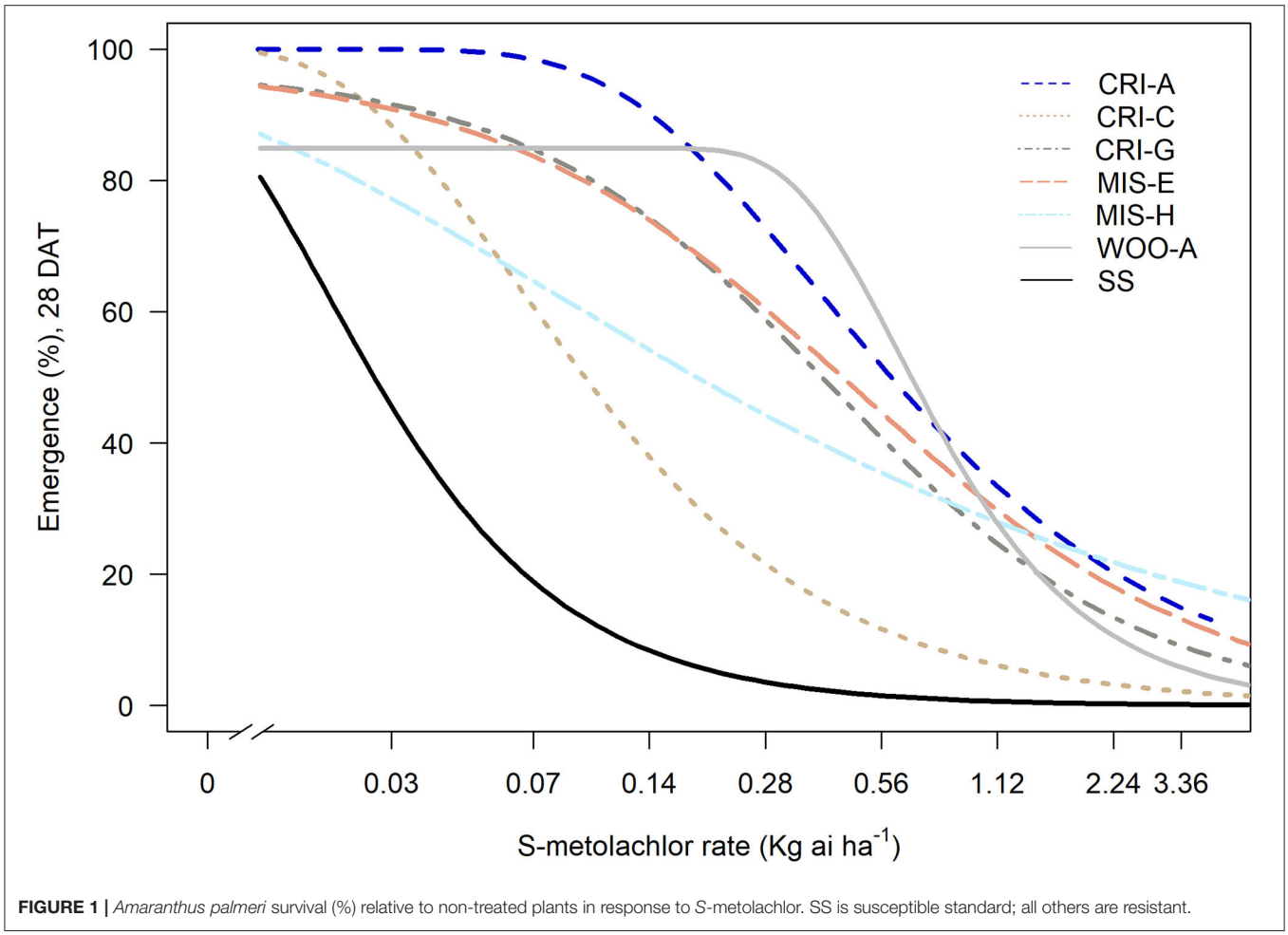

FIGURE 1 | *Amaranthus palmeri* survival (%) relative to non-treated plants in response to *S*-metolachlor. SS is susceptible standard; all others are resistant.

TABLE 1 | Resistance levels of S-metolachlor resistant *Amaranthus palmeri* populations.

Population	ED_{50} (g ai ha^{-1})[a]	R/S[b]
16WOO-A	785 (521–1049)	29
15CRI-A	593 (449–737)	22
14CRI-G	418 (244–592)	15
14CRI-C	88 (69–107)	3
14MIS-H	207 (37–376)	8
14MIS-E	467 (221–712)	17
SS	27 (21–33)	1

[a] *ED_{50}, estimated dose of S-metolachlor herbicide required to cause 50% injury. Values in parenthesis are 95% confidence intervals.*
[b] *R/S, ED_{50} of the resistant population divided by the ED_{50} of the SS population.*

comparing % root growth reduction with NBD-Cl alone across populations, SS showed the greatest inhibition (66%), followed by CRI-A (52%) and CRI-G (39%) (**Figure 2**). Considering that GST functions are not restricted to herbicide or xenobiotic detoxification, it appeared that the constitutive overexpression of some GSTs in the resistant populations afforded some protection from the phytotoxicity of NBD-Cl. The data also showed that *S*-metolachlor is more inhibitory to roots than other tissues of the seedling. This means that GST activity in roots is important in mitigating the phytotoxicity of the herbicide. Together, our data suggest that GSTs are key factors in *A. palmeri* resistance to *S*-metolachlor. A lower concentration of NBD-Cl than what was used in this study might allow better visualization of how much constitutive and inducible GST activity could impart resistance to *S*-metolachlor.

Candidate *GST* Genes

The tau and phi class GST enzymes were chosen as putative candidate genes conferring resistance to *S*-metolachlor. To select the *GST* genes, we first identified the *GST* genes involved in VLCFA inhibitor tolerance or resistance in other species, such as *Z. mays* (corn), *L. rigidum* (rigid ryegrass) and *A. myosuroides* (blackgrass) from literature (Li et al., 2017a,b; Busi et al., 2018; Dücker et al., 2020). Access to recently sequenced and deposited *A. palmeri* genome allowed retrieval of putative homologs of such functionally related *GST* genes. As specific GSTs were elicited in response to safeners in *Arabidopsis* as a main component of molecular response (DeRidder and Goldsbrough, 2006), we also recovered its homologs in *A. palmeri*. The individual analysis

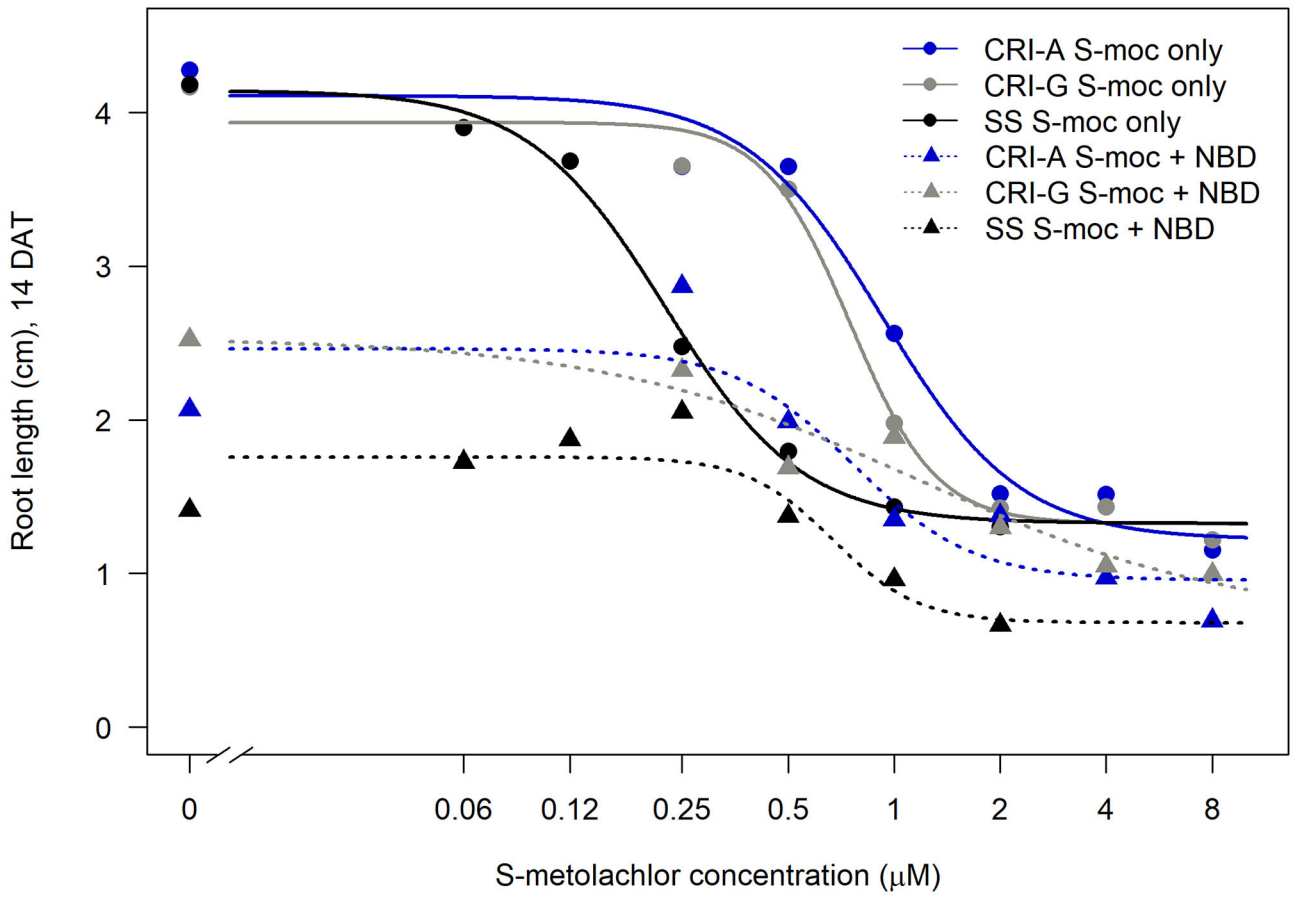

FIGURE 2 | Effect of GST inhibitor, NBD-Cl, and *S*-metolachlor on root growth of *Amaranthus palmeri* in agar-based assays. 15CRI-A and 14CRI-G are resistant populations. NBD-Cl was used at 0.25 μM. Root lengths were measured at 14 days of incubation in a growth chamber set at 30/28°C day/night temperature. The experiment was conducted twice. Each data point is the average of 25–30 plants.

of species-specific GSTs produced multiple candidate genes in *A. palmeri*. The topmost similar genes were selected using comparative analysis (BLAST). We selected five candidate genes for *S*-metolachlor detoxification. The identity of each selected candidate GSTs in *A. palmeri* and similarity with other known GSTs in different species are listed in **Table 3**. ApGSTU19 and ApGSTU18 were identified as the most probable homologs of tau class GSTs that were responsive to *S*-metolachlor or safener. ApGSTF2, ApGSTF8, and ApGSTF2like were included mainly in relation to safener inductivity.

Expression Profile of Candidate *GST*s

The expression of putative candidate *GST*s (**Table 3**) was measured in leaves and roots of survivors of 1x dose of *S*-metolachlor from resistant populations. This approach allowed the determination of the expression pattern of putative candidate *GST*s at the level of genetic transcription in different tissues, where herbicide detoxification could occur. The SS population was used as baseline. As *S*-metolachlor is a soil-applied herbicide, and our seeds were field-derived, it was impossible to obtain non-treated R plants. We could only recover confirmed R plants (the survivors); therefore, non-treated SS plants were used as control to estimate the constitutive and inductive expression level of candidate genes in R plants. Four genes, *ApGSTU19*, *ApGSTF2*, *ApGSTF8,* and *ApGSTF2like* were differentially regulated between survivors and SS control plants (**Figure 4**). The expression of *ApGSTU18* did not differ significantly between R and SS plants in roots and leaves (**Supplementary Figure 2**). The expression of *ApGSTU19* and *ApGSTF8* were constitutively upregulated in the roots of R plants. *ApGSTF8* was further upregulated three- to six-fold higher in roots in response to *S*-metolachlor in R populations. The expression of *ApGSTU19* varied from two- to six-fold in treated R populations. Interestingly, *ApGSTF8* and *ApGSTU19* were not constitutively upregulated in leaves. Survivors from 14MIS-E, 15CRI-A, and 14CRI-C showed two- to three-fold induction of *ApGSTF8* in leaves, whereas other populations did not show significant induction. Slight upregulation of *ApGSTU19* was also observed in leaves of survivors. The expression level of *ApGSTF2* and *ApGSTF2like* gene was higher in roots than leaves. These genes were not enriched in leaves of R plants; instead, its expression remained lower in leaves of R plants

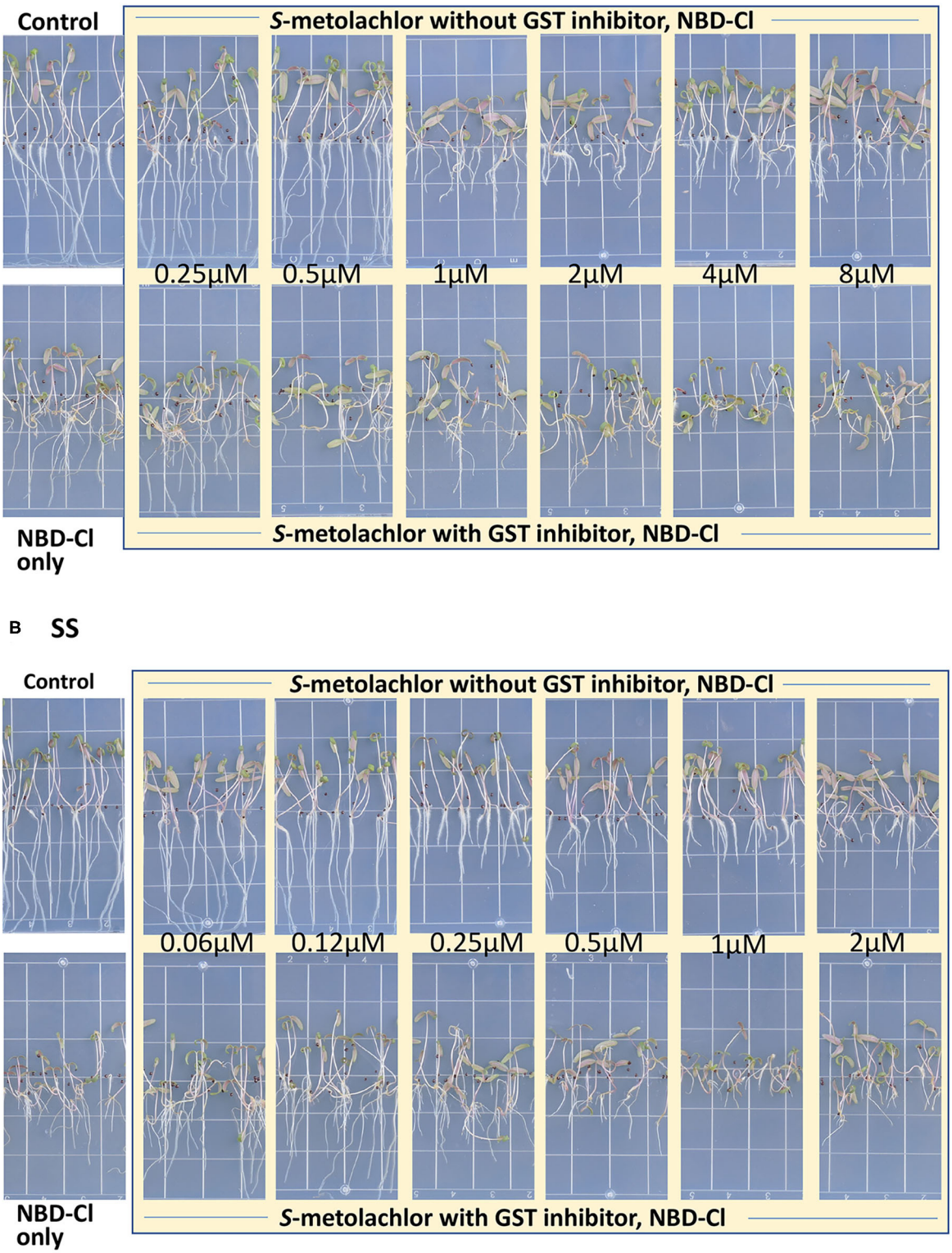

FIGURE 3 | Effect of GST inhibitor, NBD-Cl, and *S*-metolachlor on seedling growth of *Amaranthus palmeri* in agar-based assays using a representative resistant [14CRI-G(A)] and susceptible [SS (B)] population. Root lengths were measured at 14 days of incubation in a growth chamber set at 30/28°C day/night temperature. The experiment was conducted twice. The upper and lower left panels of **(A)** and **(B)** are the non-treated and NBD-Cl treated checks of resistant and susceptible populations, respectively.

TABLE 2 | Estimated GR_{50} for *S*-metolachlor tolerance in absence of NBD-Cl in *A. palmeri* populations using agar-based assay.

Accession	GR50 (uM)	Fold change
14CRI-G	0.750 (0.649–0.852)*	3.6
15CRI-A	0.929 (0.775–1.082)	4.1
SS	0.224 (0.185–0.263)	1.0

*Values within brackets are the confidence intervals ($p < 0.05$) of the estimate.

across populations compared to non-treated SS. *ApGSTF2* and *ApGSTF2like* were enriched approximately two-fold in roots of the majority of R plants upon *S*-metolachlor treatment. Survivors from 14MIS-H showed the highest induction (∼4-fold) of *ApGSTF2*, while those of 14MIS-E, 14CRI-G and 16WOO-A showed three to four-fold induction of *ApGSTF2like* gene in roots upon herbicide treatment. Overall, the comparison of tissue-specific transcript abundance showed higher expression of selected candidate genes in roots. Also, the expression level of candidate genes was not directly correlated with the level of resistance.

Phylogenetic Analysis and Substrate Binding Site Comparison

Phylogenetic analysis of selected candidate genes indicated that these genes are evolutionarily related across different species and could be functionally related (**Figure 5**). Tau class GST enzyme, ApGSTU19, is closely related to AtGST19 in *Arabidopsis* and ZmGST6 in *Z. mays*. Similarly, all phi GST enzymes included in this study are highly similar to each other. ApGSTF8 in *A. palmeri* is highly similar to ZmGST12 and ZmGST13 in *Z. mays* (**Figure 3**).

The annotation of substrate-binding site or H-site in known GST enzymes provides more detailed insights regarding critical amino acids involved in specific herbicide conjugation. Only proteins with high % sequence similarity between *A. palmeri* and other species from **Table 3** were selected for comparison of H-site. Since the *Z. mays* GST enzymes are best characterized with respect to chloroacetanilide herbicide detoxification, its H-site was used as reference to compare the similarity with candidate genes. First, the amino acid sequences of ApGSTU19 and the functionally related AtGSTU19, ZmGST6, and ZmGST5 enzymes were aligned and the H-site was annotated using information available at the NCBI Conserved Domain Database (CDD) (Lu et al., 2020). To rule out the possible contribution of any coding sequence mutation in ApGSU19 to *A. palmeri* resistance to *S*-metolachlor, nearly full-length sequence was obtained using survivors from 15CRI-A to 14CRI-G populations. There were no changes in the H-site residues compared to the reference sequence (**Supplementary Figure 3**). Analysis of the H-site of ApGSTU19 showed that the majority of residues are conserved between ApGSTU19 in *A. palmeri* and ZmGST6 and ZmGST5 in *Z. mays* (**Supplementary Figure 5**). Thus, we can predict that their biological function would be similar. The H-site of ApGSTU18 was significantly different from those of related enzymes, especially that of ZmGST34 (**Supplementary Figure 4**), which is involved in most chloroacetanilide herbicide detoxification (Li et al., 2017c). Also, the H-site of all selected phi enzymes was very different from those of ZmGST12 and ZmGST13 (**Figure 5**).

DISCUSSION

VLCFA-inhibiting herbicides are used to control a broad spectrum of broadleaf and grass weeds in various crops. Seven weed species; *L. rigidum*, *A. myosuroides*, *A. palmeri*, *Amaranthus tuberculatus*, *Avena fatua*, *Echinochloa crus-galli* var. *crus-galli,* and *Lolium perenne* ssp. *Multiflorum* have now evolved resistance to VLCFA inhibitors (WSSA-Group 15) around the world (Heap, 2021). This study aimed to understand the recent evolution of *A. palmeri* resistance to VLCFA inhibitors. Resistance to VLCFA inhibitors has evolved slowly with time, despite the sustained, high-volume usage of this group of herbicides across a large number of crops across decades. This is a testament to the difficulty of overcoming the phytotoxic effect of this type of herbicide action. The range of resistance level found in this study was wide (3-to 29-fold). For a soil-applied herbicide, this is primarily a reflection of emergence reduction, and the degree of stunting of emerged seedlings. Thus, we can equate low resistance index to less reduction in plant population. As with foliar herbicides, low frequency of resistant individuals produces low resistance indices, indicative of an early stage of resistance evolution. Populations with high resistance index are more purified, having gone through multiple cycles of reproduction of R plants and concomitant selection with the same herbicide SOA. It can also be hypothesized that a high resistance index is a latent effect of preexisting NTSR mechanisms selected by other primary herbicide selectors. In the case of Arkansas populations, this includes ALS- and PPO inhibitors. Except 16WOO-A, all other resistant populations are reported to have reduced sensitivity to multiple herbicides with different SOAs in a previous study (Salas-Perez et al., 2017). All the resistant populations used in this study showed high resistance to sub-lethal dose of *S*-metolachlor (**Supplementary Figure 1**), which is indicative of elevated baseline protection, characteristic of NTSR evolution.

Upon application of alachlor and *S*-metolachlor in crops like corn, these herbicides are found as nontoxic GSH conjugates, thus, providing tolerance to these herbicides (Shimabukuro et al., 1971; Cottingham and Hatzios, 1992; Rossini et al., 1996). These early studies suggested that enhanced metabolism driven by GSTs is the mechanism of tolerance to chloroacetanilide. Recent research on *L. Rigidum* and *A. myosuroides* resistance to pyroxasulfone and flufenacet also indicated detoxification by GSTs as the primary mechanism of resistance (Busi et al., 2018; Dücker et al., 2020). The effect of GST inhibitor on the activity of *S*-metolachlor on *A. palmeri* in our study and that of Brabham et al. (2019) indicate that GSTs endow resistance to *S*-metolachlor in *A. palmeri*. The involvement of GSTs was supported by gene expression data of *A. palmeri* GSTs in R and S plants. The four candidate *GST* genes, which showed differential response between R and S plants showed higher expression in roots than in shoots, indicating that seedlings survive because

TABLE 3 | List of selected candidate GSTs in *A. palmeri* and its amino acid sequence similarities in other species.

Putative candidate GSTs			Similarity with other species (name, protein sequence similarity)			
***A. palmeri* locus name**	**GST name (adopted from *A. thaliana*)**	**GST class**	***A. thaliana***	***Z. mays***	***L. rigidum***	***A. myosuroides***
Ap.01g001210	ApGSTU19	Tau	AtGSTU19, 69%	ZmGST6, 59% ZmGST5, 56%	LrGST-1, 37% LrGST-4, 40%	AmGST1, 33% AmGST2, 51% AmGST3, 57%
Ap.02g139000	ApGSTU18	Tau	AtGSTU18, 62%	ZmGST34, 46%	LrGST-1, 48% LrGST-4, 41%	AmGST1, 41% AmGST2, 51% AmGST3, 56%
Ap.06g223180	ApGSTF8	Phi	AtGSTF8, 50%	ZmGST12, 33% ZmGST13, 32%	-	AmGST4, 25%
Ap.05g099350	ApGSTF2	Phi	AtGSTF2, 53%	-	-	-
Ap.05g099340	ApGSTF2like	Phi	AtGSTF2, 41%	-	-	-

Protein sequences were aligned using the Clustal method and data are shown as percentage similarity.

a substantial amount of the herbicide is inactivated by GSTs as soon as it is absorbed by the roots (**Figure 4**). This was achieved by constitutive upregulation of GSTs in the roots of R plants and further induction of these GSTs in the presence of *S*-metolachlor. The suppression of GST activity by NBD-Cl, reduced the root growth more than it did the shoot growth (**Figure 2**). In S plants where GSTs were not constitutively upregulated, NBD-Cl did not affect the activity of *S*-metolachlor. Collectively, these results suggest that roots are the primary site where *S*-metolachlor detoxification by GSTs occur. This makes sense for a soil-applied herbicide with this SOA and corresponding mechanism of natural plant protection. Our findings are consistent with previous research correlating enhanced GST activity in roots of maize cultivar with tolerance to chloroacetanilide herbicides (Sari-Gorla et al., 1993; Li et al., 2017b).

Safeners are agrochemicals that physiologically increase herbicide protection by enhancing the production of selective or distinct GSTs in crops (Hatzios and Wu, 1996; DeRidder et al., 2002; Scarponi et al., 2006). Much work has been done to understand specific GSTs that are responsive to safeners using *Arabidopsis* as a model. In our study we chose GSTs responsive to safeners, benoxacor, and fluxofenim, as candidates for *S*-metolachlor degradation as these safeners are commercially used to safen grass crops from metolachlor (Davies and Caseley, 1999). These candidate genes sourced from multiple species showed evolutionary relationship with *A. palmeri* GSTs (**Figure 5**); therefore, we speculated that the *A. palmeri* homologs would retain a similar biological function in response to *S*-metolachlor, safening the weed from the herbicide.

ApGSTU19, which is a tau class GST, was differentially expressed in all R populations compared to SS population (**Figure 4**). *ApGSTU19* showed high similarity to the *AtGSTU19* in *Arabidopsis*, which was induced in response to several safeners (DeRidder et al., 2002; DeRidder and Goldsbrough, 2006; Skipsey et al., 2011). *AtGSTU19* was highly induced by benoxacor and fluxofenim, and was able to conjugate alachlor, acetochlor, and *S*-metolachlor to GSH *in vitro* (DeRidder et al., 2002). The GST enzymes contain an N-terminal thioredoxin-fold domain and a C-terminal alpha helical domain, with H-site located in a cleft between the two domains. GSH binds to the N-terminal domain called G-site, while the substrate occupies a pocket in the C-terminal domain known as H-site, together it is known as active site of GSTs. The G-site is conserved, but H-site varies between different GSTs. Active site comprising multiple overlapping hydrophobic residues within H-site allows a wide range of substrates to bind, which allows GSTs to act on diverse xenobiotics (Cummins et al., 2011). ApGSTU19 also showed high similarity, in sequence and H-site, to ZmGST5 and ZmGST6 in maize (**Figure 5**), indicating the same ability to catalyze the conjugation of GSH to *S*-metolachlor in resistant *A. palmeri* plants. Li et al. (2017a) demonstrated that *ZmGST5* and *ZmGST6* are one of the highly expressed core genes among the other *GSTs* that were found to be responsible for differential tolerance to metolachlor in tolerant maize cultivar. *In vitro* studies using recombinant maize ZmGST5 showed specificity of this GST toward *S*-metolachlor (Dixon et al., 1997, 1998). Thus, we propose that *ApGSTU19* is involved in detoxification of *S*-metolachlor in *A. palmeri*.

ApGSTU19 and *ApGSTF8* were constitutively upregulated in roots, but not in leaves, and their abundance increased in response to *S*-metolachlor in R populations. This indicates tissue-specificity geared toward protection from soil-applied herbicides, elevated baseline protection, and increased protection when challenged with herbicide. High expression of *AtGSTU19* and *AtGSTF8* was also predominant in roots and suspension culture in response to different safeners in *Arabidospis* (DeRidder et al., 2002; DeRidder and Goldsbrough, 2006; Skipsey et al., 2011). Other researchers reported similar root-specific expression profile of *ZmGST6* and other phi genes, *ZmGST12* and *ZmGST13*, accounting for crop tolerance to *S*-metolachlor (Li et al., 2017b). *ApGSTF8*, a phi class GST, was consistently the most differentially responsive among all candidate genes in roots of all R plants (**Figure 4**). *ApGSTF8* was highly similar to *AtGSTF8* (**Table 3**), which is known to be highly responsive to the safener benoxacor

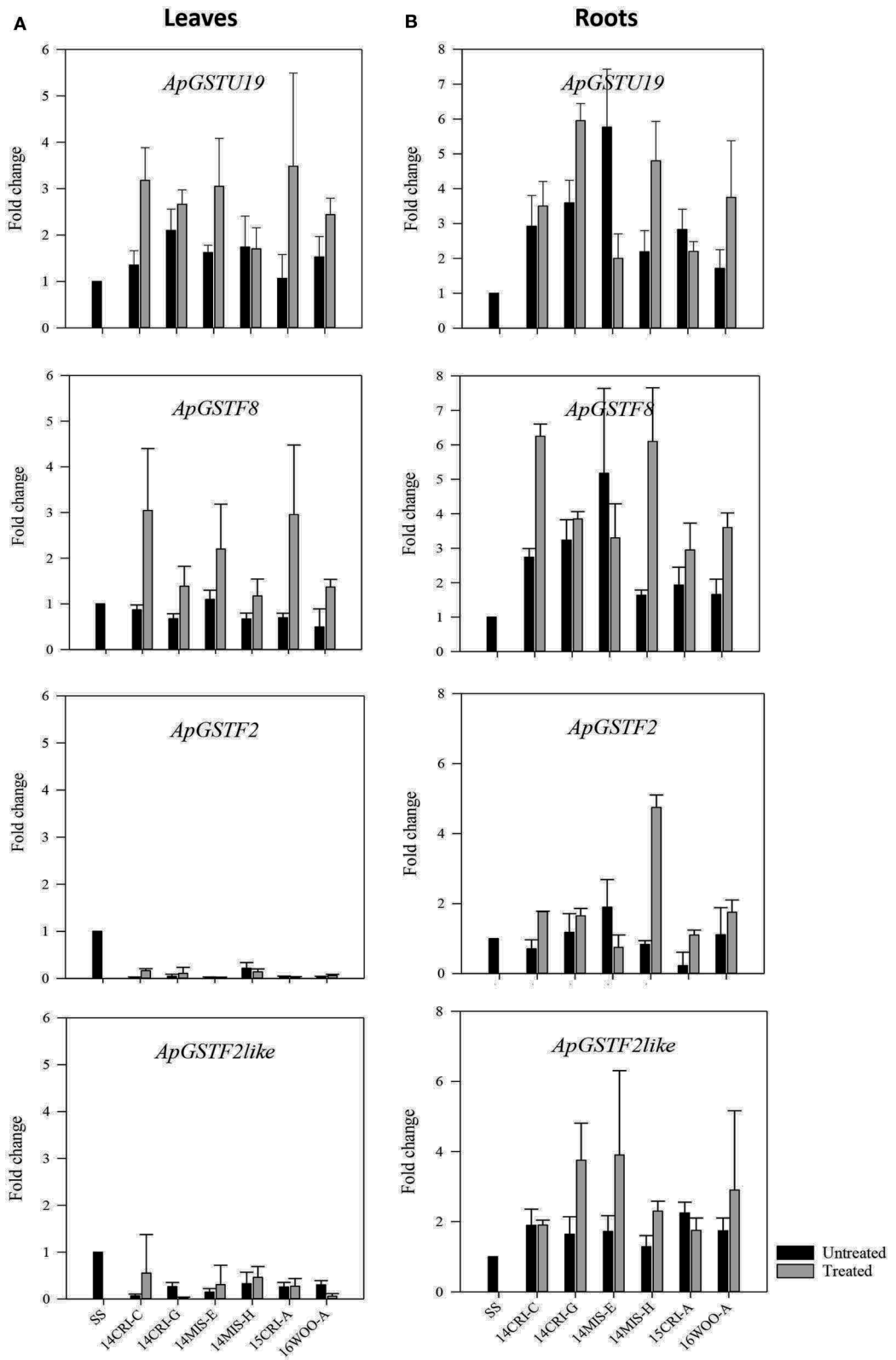

FIGURE 4 | Expression profile of candidate *GST* genes in leaves **(A)** of *S*- metolachlor-resistant and -susceptible population of *A. palmeri* and roots **(B)**. Each bar represents the relative expression (fold change) of each gene in non-treated and treated resistant plants compared to non-treated susceptible plants (SS). Data are means ± SE of two independent experiments consisting of three biological replicates except roots of treated plants.

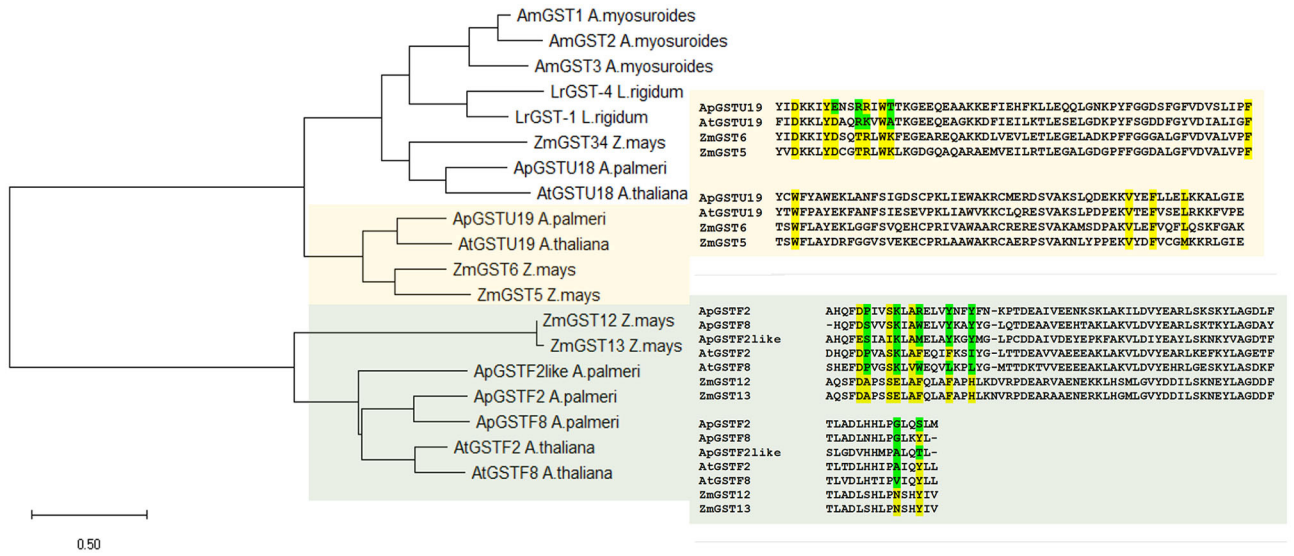

FIGURE 5 | Phylogenetic and active site analysis of selected GSTs from different species. The selected GSTs were analyzed from *Arabidopsis thaliana*, *Zea mays*, *Lolium rigidum*, *Aloperucrus myosuroides,* and *Amaranthus palmeri*. The evolutionary history was inferred using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 19 amino acid sequences, and the final dataset was composed of 285 positions. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). Active site in candidate ApGSTU19 and ApGSTF8 was identified by comparing the sequence with *Z. mays*. Highlighted in yellow is the conserved and green is the polymorphic compared to *Z. mays* active site residue. Multiple sequence alignment was carried out using uniport align tool using ApGSTU19, ApGSTF8, ApGSTF2, and ApGSTF2like from *A. palmeri*, AtGSTU19 (AT1G78380), AtGSTF2 (AT4G02520), and AtGSTF8 (AT1G02920) in *A. thaliana* and ZmGST5 (CAA73369), ZmGST6 (CAB38120), ZMGST12 (AAG34820), and ZmGST13 in *Z. mays* (AAG34821).

(DeRidder et al., 2002). In this comprehensive *Arabidopsis* study, which showed the induction of multiple GST enzymes by different safeners, the expression profile of AtGSTF8 differed from all other *GST* genes tested, showing the greatest induction by benoxacor. Benoxacor is typically added to *S*-metolachlor for use in corn to reduce injury. ApGSTF8 was also closely related to ZmGST12 and ZmGST13 enzymes (**Figure 5**), which was highly upregulated in roots of maize cultivar with differential tolerance to *S*-metolachlor (Li et al., 2017b), suggesting that ApGSTF8 may contribute to *S*-metolachlor detoxification in *A. palmeri*. The H-site analysis of ApGSTF8 enzyme did not show high similarity (**Figure 5**) to other functionally related GSTs, but its consistent differential upregulation in roots of all R plants strongly suggests involvement in *S*-metolachlor detoxification. Other phi class candidate genes, *ApGSTF2* and *ApGSTF2like* are closely related to *ApGSTF8*, but their level of expression was different among R plants from different R populations (**Figure 4**). The R populations have evolved independently of each other, considering their geographical separation; therefore, could harbor different regulation patterns of GST isozymes. AtGSTF2 is greatly induced by fluxofenim in *Arabidopsis*; thus, we expect ApGSTF2 and ApGSTF2like isozymes to react with *S*-metolachlor as well, protecting R plants. Position of *ApGSTF2* and *ApGSTF2like* gene is located adjacent to each other within the DNA sequence of *A. palmeri* genome. Our data suggests that *ApGSTF2* and *ApGSTF2like* may not be cotranscribed and are transcribed at different levels despite their close genetic linkage.

The expression of *ApGSTU18*, also a tau class GST, was not different in R plants regardless of being 50% similar to *ApGSTU19*. ApGSTU18 was chosen as a candidate GST based on its sequence similarity to LrGST1, AmGST2, and AmGST3, which were identified to confer metabolic resistance to pyroxasulfone and flufenacet in *L. rigidum* and *A. myosuroides*, respectively (**Table 3**). Sequence comparison also showed that ApGSTU18 was 46% similar to ZmGST34, which was found to be differentially regulated in maize cultivar tolerant to *S*-metolachlor. Transgenic *Arabidopsis* plants overexpressing *ZmGST34* had increased tolerance to most chloroacetanilide herbicides (Li et al., 2017c). Active site comparison between ApGSTU18 and ZmGST34 showed minimal identity (**Supplementary Figure 4**). Therefore, it is possible that the differential response of *ApGSTU18* and *ApGSTU19* to *S*-metolachlor in *A. palmeri* may lie in the difference between the topology of the substrate binding site.

The co-ordinated up-regulation of genes encoding candidate GST enzymes showed substantial similarity with those induced by safeners in *Arabidopsis*. Although treatment with various safeners failed to impart tolerance to chloroacetanilide herbicides in *Arabidopsis* (DeRidder and Goldsbrough, 2006), eliciting the expected safener response in resistant *A. palmeri* plants indicates strong association between the function of these candidate GSTs and metolachlor detoxification. Structural difference among GSTs certainly alters reactivity to *S*-metolachlor and affects resistance level. Differential resistance level to *S*-metolachlor in

A. palmeri could also be due to modifications in components of phase III detoxification. Although all candidate GSTs are differentially regulated in R plants compared to S plants, the specificity of an individual GST toward *S*-metolachlor should be tested either using heterologous expression system or *in vitro* assay to show, unequivocally, that these GSTs are responsible for *S*-metolachlor detoxification in *A. palmeri*. Transcriptome and metabolism studies are warranted.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

GR designed and conducted the candidate GST analysis, performed the molecular biology experiments, analyzed the data, and wrote the manuscript. MN performed the herbicide dose-response and GST-inhibitor assays and participated in the manuscript preparation. LB contributed to the molecular biology experiments. RS-P participated in field sampling of Palmer amaranth populations with NR-B. RS-P conducted the first general screening for *S*-metolachlor resistance and characterized the resistance profile of populations used in this study. NR-B conceived the whole Palmer amaranth project, obtained funding for the project, organized and led the collection of Palmer amaranth samples and field histories, conceptualized the phenotyping and molecular biology experiments, directed the research implementation, and worked with GR in manuscript writing and revisions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.652581/full#supplementary-material

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frontiers in Plant Science

REVIEW
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Dinitroaniline Herbicide Resistance and Mechanisms in Weeds

Jinyi Chen[1,2], Qin Yu[1], Eric Patterson[2], Chad Sayer[3] and Stephen Powles[1]*

[1] Australian Herbicide Resistance Initiative (AHRI), School of Agriculture and Environment, University of Western Australia (UWA), Perth, WA, Australia, [2] Department of Plant, Soil, and Microbial Sciences, Michigan State University, East Lansing, MI, United States, [3] Nufarm Limited, Melbourne, VIC, Australia

Dinitroanilines are microtubule inhibitors, targeting tubulin proteins in plants and protists. Dinitroaniline herbicides, such as trifluralin, pendimethalin and oryzalin, have been used as pre-emergence herbicides for weed control for decades. With widespread resistance to post-emergence herbicides in weeds, the use of pre-emergence herbicides such as dinitroanilines has increased, in part, due to relatively slow evolution of resistance in weeds to these herbicides. Target-site resistance (TSR) to dinitroaniline herbicides due to point mutations in α-tubulin genes has been confirmed in a few weedy plant species (e.g., *Eleusine indica*, *Setaria viridis,* and recently in *Lolium rigidum*). Of particular interest is the resistance mutation Arg-243-Met identified from dinitroaniline-resistant *L. rigidum* that causes helical growth when plants are homozygous for the mutation. The recessive nature of the TSR, plus possible fitness cost for some resistance mutations, likely slows resistance evolution. Furthermore, non-target-site resistance (NTSR) to dinitroanilines has been rarely reported and only confirmed in *Lolium rigidum* due to enhanced herbicide metabolism (metabolic resistance). A cytochrome P450 gene (CYP81A10) has been recently identified in *L. rigidum* that confers resistance to trifluralin. Moreover, TSR and NTSR have been shown to co-exist in the same weedy species, population, and plant. The implication of knowledge and information on TSR and NTSR in management of dinitroaniline resistance is discussed.

Keywords: dinitroaniline herbicides, trifluralin (herbicide), target-site resistance, tubulin mutations, non-target-site resistance, metabolic resistance

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Joel Torra,
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Rafael De Prado,
University of Córdoba, Spain
Marcos Yanniccari,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

***Correspondence:**
Qin Yu
qin.yu@uwa.edu.au

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INTRODUCTION

Weeds are a major threat to global food security. Weeds cause tremendous economic loss to agriculture by competing with crops for light, nutrition and water, and decreasing crop yield (Oerke, 2006). Herbicides are vital tools in controlling weeds, saving both time and labor, which in turn contributes immensely to global food production (Shaner, 2014). The first synthetic herbicide, 2,4-D, was produced in 1941 by Robert Pokorny and it is still being used today for broad-leaf weed control (Stephenson et al., 2001). After its discovery, new herbicide "modes of action" have been introduced approximately every 3 years, leading to the current availability of about 20 known modes of action.

However, persistent global herbicide application on large weed populations has resulted in the evolution of herbicide resistant weed populations. Since the first well documented herbicide resistant case for triazines in 1970 (Ryan, 1970), the total number of herbicide-resistant weed

species has increased dramatically. A conservative estimate shows herbicide resistance has evolved in 262 weed species across 71 countries (Heap, 2021). What is worse, the number of herbicide resistant weed is still increasing, whereas development of new modes of action herbicides has been decreasing since 1991 (Duke, 2012).

With ever increasing herbicide resistance, understanding resistance mechanisms provides scientists and agronomists a theoretical framework to better control, mitigate, and manage herbicide resistant populations. This review focuses on the dinitroaniline herbicides and encompasses 1) the development of dinitroaniline herbicides, 2) resistance evolution and mechanisms, 3) inheritance, 4) potential fitness cost and 5) discussions on possible tactics to mitigate dinitroaniline resistance evolution.

DEVELOPMENT OF DINITROANILINE HERBICIDES

Dinitroanilines represent a class of chemicals with a structure containing two nitro groups and an aromatic amine, aniline. Originally discovered in evaluations of dyes and dye chemical synthesis intermediates, dinitroanilines grew to be widely used in agriculture, industry and medical science (Dekker, 1999). In agriculture, dinitroanilines are mainly used as pre-emergence herbicides to control grass and some broadleaf weeds (Parka and Soper, 1977). The commercialized dinitroaniline herbicides so far include trifluralin, pendimethalin, ethalfluralin, oryzalin, butralin, benefin/benfluralin and prodiamine. The first dinitroaniline herbicide, trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine, **Figure 1**), was commercialized in the 1960s in the United States (Grover et al., 1997; Timmons, 2005). Originally it was used in soybean fields by pre-plant soil-incorporation for grass weed control (Epp et al., 2017). Later on, trifluralin was introduced into Latin America and Asia Pacific and extensively used in sugarcane and soybean in Brazil (Lima et al., 2018), and Australian cereal and legume fields (Jolley and Johnstone, 1994). With the introduction of newly developed, highly efficient post-emergence ALS- and ACCase-inhibitors in 1980s, trifluralin usage declined and the trifluralin market was significantly replaced by these newer herbicides. However, due to the rapid resistance evolution to these newer, post-emergence herbicides and the adoption of no-till or reduced tillage techniques for soil and moisture conservation, trifluralin has resurged in many markets. According to data from the Brazilian Institute of Environment and Renewable Natural Resources, sales of trifluralin comprised 1,887 tons in 2019 (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), 2020). In the United States, trifluralin was among the 25 most used pesticides in agriculture, and the estimated usage ranged from 1361 to 3175 tons in 2012 [Environmental Protection Agency (EPA), 2021]. In recent years trifluralin and pendimethalin have been the two most significant dinitroaniline herbicides used, estimated to represent a global farm gate value (the dollar amount of sales of product made to the actual farmer) of $USD 525 million. When considering trifluralin/pendimethalin, over 30% of the farm gate value is within the Australian and North American markets, and 50% of farm gate value is in cereals, cotton, vegetables and soybean. Controlling weed populations resistant to other herbicide chemistries is generally accepted as a significant factor driving dinitroaniline herbicide use in these regions and crops.

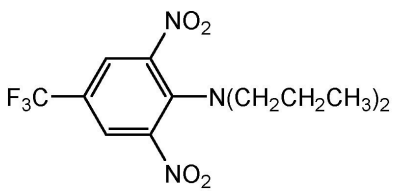

FIGURE 1 | Trifluralin chemical structure.

BEHAVIOR AND MODE OF ACTION OF DINITROANILINE HERBICIDES

Dinitroanilines have low water solubility (20°C in water, 0.22 mg/L for trifluralin, and 0.33 mg/L for pendimethalin). According to Environmental Protection Agency (EPA) 1996, trifluralin ranks as moderate to highly toxic for aquatic animals (fish and invertebrates) [Environmental Protection Agency (EPA), 1996]. Most dinitroaniline herbicides are highly volatile. At 25°C, the volatility of trifluralin is 9500 kPa, and pendimethalin, 1940 kPa (versus 3.17 kPa for water) (Congreve and Cameron, 2014). Dinitroanilines are subject to decomposition due to photodegradation (Wright and Warren, 1965), and its effectiveness is greatly affected by its soil incorporation depth (Savage and Barrentine, 1969; Spencer and Cliath, 1974). Therefore, dinitroaniline herbicides need to be incorporated with surface soil to minimize volatilization and photodegradation loss. Particularly, in Australia, the "incorporate by sowing" technique uses a knife point seeder to throw soil into the inter-row to cover the herbicide and reduce loss due to volatilization (Ashworth et al., 2010). When within the soil, dinitroanilines have strong binding coefficient (K_{oc}, if K_{oc} > 4,000, non-mobile) with the soil (K_{oc} pendimethalin = 17,581, K_{oc} trifluralin = 15,800) (Helling, 1971, 1976; Congreve and Cameron, 2014), and slow microbial degradation. Microbial degradation is the primary breakdown route; however, persistence is generally long, often resulting in rotational crop limitations (Congreve and Cameron, 2014).

Due to low solubility in soil moisture and strong soil-binding, dinitroanilines enter the germinating seedlings primarily *via* gaseous absorption through the root, coleoptile node or hypocotyl, upon contact with the herbicide (Congreve and Cameron, 2014). Trifluralin soil-borne vapor plays an important phytotoxic role especially to plant roots (Barrentine and Warren, 1971; Charles and Richard, 1972). In early research on dinitroaniline herbicides uptake and

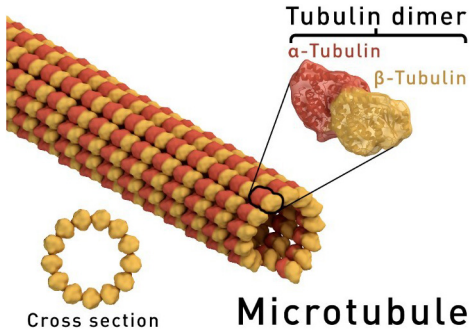

FIGURE 2 | Simulative structure of a microtubule. The ring shape depicts a microtubule in cross-section, showing the 13 protofilaments surrounding a hollow centre (https://goo.gl/images/BKJ9m3, under the Creative Commons Attribution-Share Alike 4.0 International license).

translocation, it was found that in both monocots and dicots, the translocation of ^{14}C-profluralin radioactivity to plant tops was very limited, while ^{14}C-dinitramine was more readily translocated throughout the plant, and higher temperature (38°C) could help enhance the translocation to leaves (Hawxby, 1974). Limited trifluralin translocation to the aerial portions of the plant was reported in soybean and cotton plants (Strang and Rogers, 1971), and in *Alopecurus aequalis* seedlings (Hashim et al., 2012).

Dinitroanilines target microtubules, which, together with microfilaments and intermediate filaments, are major components of the cytoskeleton. Microtubules are hollow cylinders, about 25 nm in diameter, that are comprised of α- and β-tubulin heterodimers (usually 13 protofilaments in eukaryotic cells) (**Figure 2**; Tilney et al., 1973; Desai and Mitchison, 1997; Downing and Nogales, 1998a,b). The α- and β-tubulins, each with a molecular weight of 50,000 daltons, share 36–42% amino acid sequence identity (Little and Seehaus, 1988). Microtubules perform various functions at different stages of cellular activity. During interphase, microtubules are critical for orchestrating cell wall synthesis in plant cells. Also, microtubules are anchored to the plasma membrane, forming cortical microtubules to help support cell shape. During mitosis, the bipolar spindle apparatus is comprised of microtubules and is capable of correctly positioning chromosomes to the cell midplane and then guiding separated chromatids to opposite ends of the cell (Shaw and Vineyard, 2014). To realize their mobile function(s), microtubules are required to be in a dynamic balance. There is a positive (+) and negative (−) end for microtubules; the positive end assembles the heterodimers using GTP, while the negative end dissociates into heterodimers. With balanced polymerization and de-polymerization, mitosis proceeds normally.

Dinitroanilines disrupt microtubule function by binding with unpolymerized tubulin heterodimers. *In vitro* analyses of *Chlamydomonas reinhardtii* tubulin showed specific binding with trifluralin, indicating that tubulin is the primary subcellular target of dinitroaniline action (Stokkermans et al., 1996). Similar for tubulin from *Zea mays* L., dinitroanilines bind to the unliganded α/β-tubulin heterodimers to form a herbicide-tubulin complex, and, with addition of the complex to the positive end of the growing microtubule, further elongation of the microtubule ceases (Hugdahl and Morejohn, 1993). Concomitantly, due to depolymerization of microtubules from the negative end, the microtubules become progressively shorter, eventually leading to their complete dissociation (Cleary and Hardham, 1988). When this occurs, mitosis is disturbed and mitotic cells are arrested in telophase. This is also supported by the cytological studies showing arrested mitosis at prometaphase due to loss of spindle microtubules, and formation of isodiametric cells in the elongation zone due to loss of cortical microtubules after trifluralin treatment (Vaughn, 1986). The cessation of cell division causes treated seedlings to exhibit swollen and stunted root symptoms (Lignowski and Scott, 1971). The affected seedlings either cannot emerge from the soil or there is no growth after emergence.

Interestingly, dinitroaniline herbicides target microtubules from both plants and protists, but not from animals or fungi (Chan and Fong, 1994; Bell, 1998; Dempsey et al., 2013), likely due to differences in binding affinities for animal tubulins. Oryzalin was found to bind *in vitro* to tubulins isolated from maize and *Chlamydomonas eugametos*, but not to purified tubulins from bovine brain tissue (Strachan and Hess, 1983; Morejohn et al., 1987; Hugdahl and Morejohn, 1993). Carrot is a notable exception and shows natural tolerance to dinitroaniline herbicides. Immunofluorescence and electron microscopy indicated that the microtubules of carrot roots were unaffected by dinitroaniline treatment (Vaughan and Vaughn, 1988).

Dinitroaniline herbicides inhibit the plant tubulin protein family, which is encoded by a multi-gene family comprised of several α- and β-tubulin genes. In the model plant *Arabidopsis thaliana*, there are at least six expressed α-tubulin genes and nine expressed β-tubulin genes (Snustad et al., 1992; Kopczak et al., 1992). In rice, there are three α-tubulin transcripts and eight β-tubulin genes reported (Qin et al., 1997; Yoshikawa et al., 2003). Similarly in *E. indica*, four β-tubulin and three α-tubulin isoforms have been identified (Waldin et al., 1992; Yamamoto et al., 1998; Yamamoto and Baird, 1999), whereas in *Setaria viridis,* two α- and two β-tubulin genes have been isolated (Waldin et al., 1992; Délye et al., 2004). In cross-pollinated *L. rigidum*, there are at least four α-tubulin isoforms, TUA1, TUA2, TUA3, and TUA4, but the number of transcripts coding for each isoform varies among individual/population (Chen et al., 2020b).

Crop selectivity of dinitroaniline herbicides is possible using physical herbicide positioning between crop and weed seeds in the soil. Crop seeds are beneath the layer of soil containing the dinitroaniline herbicide while the smaller annual weed seeds germinate on or near the soil surface. Furthermore, the trifluralin molecule has some species-specific selectivity and is generally more toxic to grass species (wheat, oats, barley, and rice) than broadleaf species (cotton, soybean, pea, and cucumber) (Barrentine and Warren, 1971). As trifluralin can be trapped in lipids, it is hypothesized that selective phytotoxicity of trifluralin in young seedlings is determined,

in part, by the amount of endogenous lipids available to trap trifluralin and keep it from its site of phytotoxic action (Hilton and Christiansen, 1972).

RESISTANCE EVOLUTION TO DINITROANILINE HERBICIDES

Dinitroaniline herbicides have been used for more than 50 years; however, limited cases have been documented for dinitroaniline herbicide resistance in weedy species. Globally, thus far, populations of only seven weed species have been identified to be resistant to dinitroaniline herbicides (Heap, 2021): *E. indica*, *S. viridis*, *Amaranthus palmeri*, *Alopecurus myosuroides*, *Lolium rigidum*, *Poa annua* and *Alopecurus aequalis*. These resistant weed populations are mostly in Australia, America, and Japan, where dinitroaniline herbicides have been intensively used.

E. indica from South Carolina was among the first reports of dinitroaniline resistance following about a decade's persistent application (Mudge et al., 1984). Subsequently, two more dinitroaniline-resistant biotypes of *E. indica* (i.e., resistant and intermediate resistant) were characterized (Vaughn et al., 1990). Later resistance was found in populations of *A. palmeri* and *P. annua* from other parts of the Carolinas and Georgia (Gossett et al., 1992; Lowe et al., 2001; Isgrigg et al., 2002).

Setaria viridis is the major dinitroaniline-resistant weed species reported in Canada. Trifluralin-resistant *S. viridis* populations were first found in southern and southwestern Manitoba (Morrison et al., 1989), and the resistance persisted for at least 7 years (Andrews and Morrison, 1997). In United Kingdom, one chlorotoluron-resistant *A. myosuroides* population was reported to metabolize and thus resist pendimethalin but not trifluralin (James et al., 1995). In Japan, trifluralin-resistant *A. aequalis* populations were identified after more than two decades of dinitroaniline usage (Hashim et al., 2012).

Lolium rigidum from Australia is prone to herbicide resistance evolution, and dinitroaniline herbicides are no exception (McAlister et al., 1995). Periodic herbicide resistance surveys in Australian agricultural areas show that more than 50% surveyed populations from South Australia and Victoria, and 26% from the Western Australia have become trifluralin resistant (Boutsalis et al., 2006; Owen et al., 2007, 2014). Among others, this is likely ascribed to reduced herbicide control efficacy due to high *L. rigidum* seed numbers, low herbicide doses (caused by dry environments, microbial metabolism of the herbicides etc.), and perhaps more importantly, development of metabolism-based cross-resistance in *L. rigidum* populations selected by other herbicides (e.g., Han et al., 2020). With increasing usage of trifluralin, more trifluralin-resistant *L. rigidum* populations are expected.

Relatively slow evolution of dinitroaniline herbicide resistance in weeds is likely due to several reasons: (1) Plants surviving the pre-emergence herbicide treatment early in the season can still be controlled by the application of herbicides from different modes of action, applied post-emergent, as well as by any other control methods implemented; (2) Use mixtures of pre-emergence herbicides with different modes of action significantly improves weed control efficacy (Soltani et al., 2020); and (3) Other factors like the existence of multiple target isoforms, genetic control mechanisms, and fitness costs associated with resistance alleles, as discussed below.

RESISTANCE MECHANISMS TO DINITROANILINE HERBICIDES

Generally, herbicide resistance mechanisms can be divided into target-site resistance (TSR) and non-target-site resistance (NTSR). TSR refers to resistance caused by the changes in herbicide target protein including mutation, duplication and overexpression, while NTSR includes all resistance mechanisms bypassing the TSR, primarily anything that reduces the amount of herbicide reaching the target protein such as alterations in absorption, translocation or metabolism (Powles and Yu, 2010). TSR is relatively easy and straightforward to study when the target protein is not part of a multi gene family and in diploid plant species, whereas unraveling NTSR mechanisms is more technically challenging and requires a more in depth understanding of the weed's genetics and physiology.

TSR Mechanisms to Dinitroaniline Herbicides

Dinitroanilines mainly target α- and β-tubulin in protists and higher plants. Resistance mutations identified in protists offer valuable reference. In protists, the first α-tubulin mutation (Tyr-24-His) conferring resistance to dinitroanilines was identified in a single-celled algae: *Chlamydomonas reinhardtii* (James et al., 1993). Later, more resistance mutations were documented in *Toxoplasma gondii* (Morrissette et al., 2004; Ma et al., 2007, 2008; Lyons-Abbott et al., 2010) and *Tetrahymena thermophila* (Lyons-Abbott et al., 2010). In weedy plants, the first resistance-endowing tubulin mutation (Thr-239-Ile) was characterized in *E. indica* (Anthony et al., 1998; Yamamoto et al., 1998). Subsequently, the same mutation was also identified in the protist *T. gondi* (Morrissette et al., 2004) and in weedy species *S. viridis* (Délye et al., 2004) and more recently in *L. rigidum* (Chen et al., 2018b). There are other resistance mutations identified in other plants (**Table 1**) and some of them are shared by plants and protists (**Table 2**). Thus far, there is a greater number of resistance mutations identified in protists than in plants (Morrissette et al., 2004; Pham and Morrissette, 2019), which may be due to their shorter life cycles, simple genome composition and laboratory-based forced selection/evolution in protists. Given the equal sensitivity to dinitroaniline herbicides in protists and in plants, more resistance-endowing mutations discovered in protists are expected to be eventually detected in plants, as dinitroaniline selection pressure continues and/or increases.

Target-site resistance in the obligatory cross-pollinated *L. rigidum* is more complicated when compared with self-pollinated weed species, mostly due to *Lolium* genetic diversity. At least four α-tubulin isoforms (named TUA1–TUA4) have been identified in plants from two *L. rigidum* populations,

TABLE 1 | Mutations in α- and β-tubulin identified in field-evolved dinitroaniline-resistant plants.

Mutation Site	Organism	Wild type amino acid	Substituted amino acid	References
α-tubulin				
125	*A. aequalis*	Leu	Met	Hashim et al., 2012
136	*A. aequalis S. viridis*	Leu	Phe	Délye et al., 2004
202	*A. aequalis L. rigidum*	Val	Phe	Hashim et al., 2012; Fleet et al., 2017; Chen et al., 2018b
239	*E. indica S. viridis L. rigidum*	Thr	Ile	Anthony et al., 1998; Délye et al., 2004; Yamamoto et al., 1998; Fleet et al., 2017; Chen et al., 2018b
243	*L. rigidum*	Arg	Met/Lys	Chu et al., 2018
268	*E. indica*	Met	Thr	Yamamoto et al., 1998
390 + 442*	*L. rigidum*	Arg, Asp	Cys, Glu	Chen et al., 2020b
β-tubulin				
241	*Poa annual L.*	Arg	Lys	Lowe et al., 2001

**390 + 442 double mutation confers resistance to dinitroaniline herbicides.*

TABLE 2 | Common α-tubulin mutations and mutation sites shared by the protozoan *Toxoplasma* and higher plants.

Site	Amino acid substitution	Tubulin Gene (Isoform)	Organism	References
136	Leu136Phe	AAA21350.1 (α-tubulin)	*T. thermophila*	Lyons-Abbott et al., 2010
	Leu136Phe	XP_002364807.1 (α1-tubulin)	*T. gondii*	Morrissette et al., 2004
	Leu136Phe	**BAJ06363.1 (TUA1)**	***A. aequalis***	**Hashim et al., 2012**
	Leu136Phe	**CAE52515.1 (α2-tubulin)**	***S. viridis***	**Délye et al., 2004**
239	Thr239Ile	XP_002364807.1 (α1-tubulin)	*T. gondii*	Morrissette et al., 2004
	Thr239Ile	**AAC05717.1 (TUBA1)**	***E. indica***	Yamamoto et al., 1998; Anthony and Hussey, 1999
	Thr239Ile	**MT514937 (TUA4)**	***L. rigidum***	**Fleet et al., 2017; Chen et al., 2018b**
	Thr239Ile	**CAE52515.1 (α2-tubulin)**	***S. viridis***	**Délye et al., 2004**
268	Met268Thr	XP_002364807.1 (α1-tubulin)	*T. gondii*	Ma et al., 2007
	Met268Thr	**AAC05717.1 (TUBA1)**	***E. indica***	**Yamamoto et al., 1998**
243	Arg243Cys	XP_002364807.1 (α1-tubulin)	*T. gondii*	Morrissette et al., 2004
	Arg243Ser	XP_002364807.1 (α1-tubulin)	*T. gondii*	Morrissette et al., 2004
	Arg243Lys	**MT514937 (TUA4)**	***L. rigidum***	**Chu et al., 2018**
	Arg243Met	**MT514937 (TUA4)**	***L. rigidum***	**Chu et al., 2018**

Information about plant species is in bold.

with resistance-endowing mutations occurring largely in TUA3 and TUA4, according to our sequencing results in several resistant populations (Chen et al., 2018b, 2020b; Chu et al., 2018). Moreover, the same α-tubulin isoform from a single plant can be encoded by transcripts with differences only in untranslated regions (UTR), and thus adding another layer of complexity to tubulin gene sequencing and cloning (Chen et al., 2020b). In addition, there exists a substantial amount of synonymous mutation among α-tubulin transcripts encoding the same isoform from different populations, which challenges the success of PCR amplification by using a single primer pair (Chen et al., 2020b). This discovery suggests care should be taken when attempting to definitively determine the presence or absence of TSR mutations if the herbicide target is derived from a multiple gene family, even in a diploid species (i.e., it is similar to herbicides that target single genes in polypoid species). Also, it is worth noting that, due to genetic variation in different populations or even individuals within a population, one cannot entirely rely on RNA-seq information collected from a small number of plants. In these cases, validation of RNA-seq data by isoform-specific PCR is critical, as errors in alignment and assembly can occur when assembling highly similar sequences from the same gene family.

Due to the redundancy of function in a multi-gene family like tubulin, a greater level of diversity in resistance mutations is possible at both the population and individual level. For instance, at least three TUA4 mutations were previously identified in *L. rigidum* population M4/16, including Val-202-Phe, Thr-239-Ile, and Arg-243-Lys/Met (Chen et al., 2018b; Chu et al., 2018), and individuals with different combinations of mutations (e.g., 202 + 243, 202 + 239, or 239 + 243) have also been identified (Fleet et al., 2017; Chu et al., 2018) most likely due to obligate out-crossing in *Lolium*. More than two resistance alleles can exist in a single plant due to presence of multiple gene copies or transcripts. For instance, a plant homozygous for the Val-202-Phe mutant alleles in TUA4 also has the Arg-390-Cys + Asp-442-Glu resistance allele in TUA3 (Chen et al., 2020b). Nevertheless, the frequency of resistance mutations varies even among individuals of a population. In one study, 39 resistant *L. rigidum* plants were analyzed. The

Val-202-Phe mutation was most commonly found (90%), with the Arg-243-Met mutation occurring in only 7% of screened plants. Furthermore, no plants were identified as homozygous for the Arg-243-Met mutation (Chen et al., 2018b; Chu et al., 2018). This is likely related to the herbicide selection pressure and fitness penalties associated with this mutant allele (see below). As more resistant populations/plants are analyzed, a clearer picture of resistance mutation frequency will be determined in *L. rigidum,* which may be echoed in other plant species.

Unlike TSR to other herbicides (e.g., glyphosate) involving target gene duplication/overexpression, the chance is rare for evolution of such TSR mechanisms to dinitroaniline herbicides in weeds, unless both α- and β-tubulin are involved. This is expected, as the amount of α- and β-tubulin isoforms are in dynamic balance for cell vitality, and overexpression of either of the two may be lethal (Anthony and Hussey, 1998). In addition, tubulins are structural proteins and constitutively expressed in abundance, thus overexpression is a less-likely mechanism for conferring resistance.

NTSR Mechanisms to Dinitroaniline Herbicides

No difference in herbicide uptake and/or translocation has so far been ascribed to dinitroaniline resistance (McAlister et al., 1995; Hashim et al., 2012), although dinitroaniline translocation patterns are herbicide-, weed species-, and experiment-dependent (Hawxby, 1974). This is in line with the fact that dinitroaniline herbicides are often phytotoxic to germinating seedlings and therefore little whole plant translocation of the herbicide is needed for activity. Instead, thus far, enhanced dinitroaniline metabolism (metabolic resistance) has been demonstrated as the main NTSR mechanism in studied weed species. It has been challenging to identify the major trifluralin metabolic pathway and to isolate trifluralin metabolites in plants (Probst et al., 1967; Biswas and Hamilton, 1969), and extraction and quantification of dinitroaniline metabolites is hindered by the highly volatile nature of dinitroaniline herbicides. These difficulties have meant that TSR has been the primary research focus in understanding resistance mechanisms to dinitroanilines in plants. Nevertheless, essential dinitroaniline herbicide metabolites are recently identified using yeast-expressed plant cytochrome P450 enzymes (see below) (Abdollahi et al., 2021).

There is some indirect evidence for enhanced dinitroaniline herbicide metabolism in resistant weed species. In *A. myosuroides*, a single population that can metabolize chlorotoluron (and that is cross-resistant to pendimethalin) is thought to be metabolically resistant due to oxidative degradation of the 4-methyl group in pendimethalin (James et al., 1995). In *L. rigidum*, the P450 inhibitor malathion showed a synergistic effect with pendimethalin (Tardif and Powles, 1999), and phorate [another cytochrome P450 inhibitor (Ferhatoglu et al., 2005)] can partially reverse trifluralin resistance (Busi et al., 2017). Recently, enhanced trifluralin metabolism in several *L. rigidum* populations has been identified and a diagnostic assay using ^{14}C-trifluralin established for metabolic resistance (Chen et al., 2018a). Furthermore, a cytochrome P450 gene, CYP81A10v7, has been identified and characterized from a trifluralin resistant *L. rigidum* population. When rice seedlings were transformed with a CYP81A10v7 over-expression construct, they became moderately resistant to trifluralin (Han et al., 2020). This is the first metabolic gene identified that is clearly associated with the evolution of trifluralin resistance. It should be noted that the resistance level in the transgenic rice is lower than what was observed in the resistant *L. rigidum* plants. Recently, the CYP706 family from Arabidopsis and other plant species have been demonstrated to be able to metabolize most dinitroaniline herbicides (essentially to mono- and di-oxygenated compounds), including trifluralin, pendimethalin and ethalfluralin, and thus weeds have potential for the evolution of dinitroaniline metabolic resistance if these P450 genes are selected (Abdollahi et al., 2021). Taken together, these studies affirm that metabolism is a viable NTSR mechanism to dinitroanilines in weeds, though much remains to be revealed.

It is common for weeds to evolve both TSR and NTSR to commonly used herbicides and, especially in cross-pollinated species, for those mechanisms to be stacked in the same population or in the same plants. This has been previously shown for other high-use herbicides such as glyphosate, ALS- or ACCase-inhibitors (Délye, 2013; Yu and Powles, 2014; Duke, 2019), and now in dinitroanilines. For instance, in a *L. rigidum* population (202FT), both target-site mutations and non-target-site herbicide metabolism contribute to trifluralin resistance (Chen et al., 2018a,b, 2020a,b). The prevalence of each mechanism in various populations leads us to assume that more populations containing both TSR and NTSR are to be uncovered, at least for *L. rigidum*.

GENETIC INHERITANCE OF DINITROANILINE RESISTANCE

Genetic inheritance studies of dinitroaniline resistance have been carried out in two self-pollinated resistant weed species: *E. indica* (Zeng and Baird, 1997) and *S. viridis* (Jasieniuk et al., 1994). In both cases, resistance was conferred by TSR, later revealed as the α-tubulin mutations Thr-239-Ile and Met-268-Thr in *E. indica* (Anthony et al., 1998; Yamamoto et al., 1998), and Leu-136-Phe and Thr-239-Ile in *S. viridis* (Délye et al., 2004). Interestingly, and contrary to most heredity patterns of TSR, TSR to dinitroaniline herbicides in these weed species were reported to be recessive traits.

A similar inheritance study for dinitroanilines was recently carried out in one *L. rigidum* population (202FT) (Chen et al., 2019). Plants of this population are homozygous for the Val-202-Phe mutation in TUA4. Generally, dominance of the resistance trait and gene loci contribution to herbicide resistance are rate-dependent. It was shown that at 480 g ha^{-1} trifluralin (half of the field rate), resistance is inherited as a single, recessive nuclear gene trait, similar to what has been shown in *E. indica* (Zeng and Baird, 1997) and *S. viridis* (Délye et al., 2004). However, at the lower rate of 120 g. ha^{-1} trifluralin, the resistance trait does not follow a single gene, recessive pattern, indicating other

unknown but possibly weak TSR or even metabolism-based NTSR mechanisms involved in resistance.

Given the recessive nature of TSR to dinitroaniline resistance at the field relevant rates, and dinitroanilines targeting a small nuclear gene family with multiple gene copies, it follows that dinitroaniline resistant plants should be rare. However, dinitroaniline herbicide resistance evolution in weeds should not be underestimated, especially in cross-pollinating weed species with high levels of genetic diversity. Cross-pollinated species (e.g., *L. rigidum*) are capable of accumulating numerous resistance-conferring genes/mutations, either TSR or NTSR, in single plants quickly due to the sheer number of plants and their obligate cross-pollination. Another factor that may increase rates of dinitroaniline resistance evolution is that lower-than-field-rate levels of dinitroaniline are often encountered when environmental and soil conditions are unfavorable during or shortly after herbicide application. In these situations, minor resistance genes from the standing variations can be enriched and selected for, as has been demonstrated for resistance evolution to other pre-emergence herbicides under recurrent low herbicide rate selection (Busi and Powles, 2016).

FITNESS COST OF DINITROANILINE RESISTANCE

So far, limited fitness studies on dinitroaniline herbicide resistance have been conducted. In protists, like the haploid parasite *T. gondii*, various α-tubulin mutations (at positions 136, 239, 243 and 268) confer dinitroaniline resistance at a cost to microtubule function (Ma et al., 2008). Interestingly, when α-tubulin mutants (Phe-52-Tyr) were grown without dinitroanilines, they spontaneously acquired a secondary mutation (Ala-273-Val or Asp-367-Val) which increased parasite fitness, although resistance level of mutants with the double mutation also decreased (Ma et al., 2007). Similarly, more secondary mutations were detected in parasites including the Phe-52-Tyr or Glu-142-Ser α-tubulin mutations, which reduced resistance but helped improve fitness (Ma et al., 2008). It remains to be determined if the compensating tubulin mutations also occur in other species, including higher plants.

In higher plants, a fitness cost study using near-isogenic *Setaria* lines conducted in the greenhouse and in the field showed that, without herbicide treatment, plants homozygous for the Thr-239-Ile mutation were smaller and had lower 1,000-grain weight (Darmency et al., 2011). In *L. rigidum*, potential fitness cost on plant biomass was observed in plants homozygous for the Arg-243-Met mutation (Chu et al., 2018). Notably, the frequency of the Arg-243-Met mutation in the field-collected *L. rigidum* population (M4/16) was found to be low. Only two 243-Met heterozygotes were uncovered in 39 resistant plants analyzed, while homozygous resistant plants were not detected. This disequilibrium may result from a severe fitness cost of this mutation. Controlled greenhouse crosses of Arg-243-Met *L. rigidum* heterozygotes produced

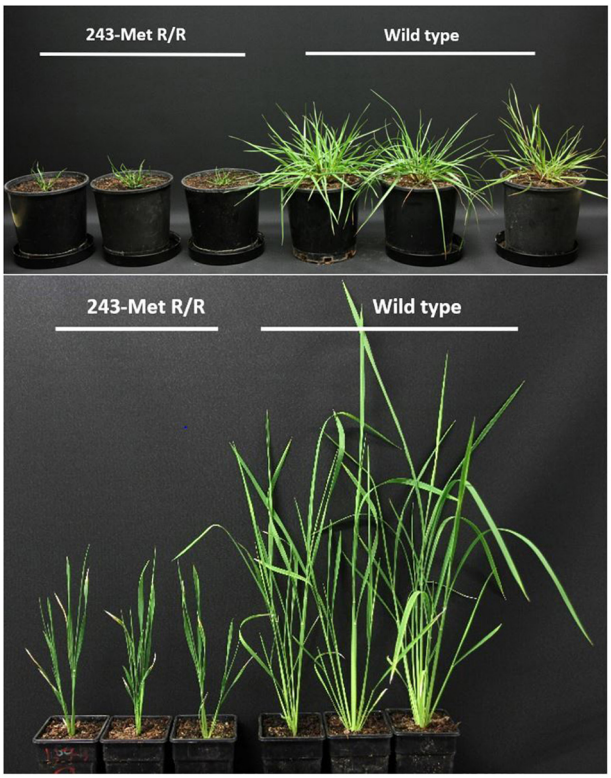

FIGURE 3 | Helical growth of *Lolium rigidum* (top picture, on the left) and transgenic rice (T2, bottom picture, on the left) homozygous for the α-tubulin Arg-243-Met mutation (243-Met R/R) in comparsion to the norma growth of corresponding wild type plants (on the right). Photos were taken 42 and 30 days after *L. rigidum* and rice transplanting, respectively.

Arg-243-Met homozygotes exhibiting severe dwarfism and right-handed helical growth (**Figure 3**). Furthermore, homozygous rice plants transformed with the *Lolium* Arg-243-Met mutant tubulin gene also exhibited dwarf and helical growth (**Figure 3**), indicating that this Arg-243-Met α-tubulin mutation confers aberrant plant morphology, although the cellular basis of these abnormalities remains to be investigated.

Preliminary observation of *L. rigidum* indicates a severe cost to TSR based dinitroaniline resistance in plant vegetative growth (Chu et al., 2018). In both *L. rigidum* and transgenic rice plants heterozygous for the 243-Met mutant allele, no altered growth phenotypes have been observed. This suggests that both the cost of resistance and the resistance itself are recessive. This is similar to the fitness cost associated with the target-site mutations (e.g., Asp-2078-Gly mutation) to ACCase-inhibiting herbicides (Menchari et al., 2008; Vila-Aiub et al., 2015) and TIPS to glyphosate (Han et al., 2017). These growth abnormalities associated with the homologous 243-Met mutant allele starkly contrast with the lack of visible growth defects shown for the most commonly identified α-tubulin mutation, Val-202-Phe in *L. rigidum*, similar to the most popular ACCase resistance mutation: Ile-1781-Leu (Wang et al., 2010). This may explain the much higher frequency of the Val-202-Phe than the Arg-243-Met

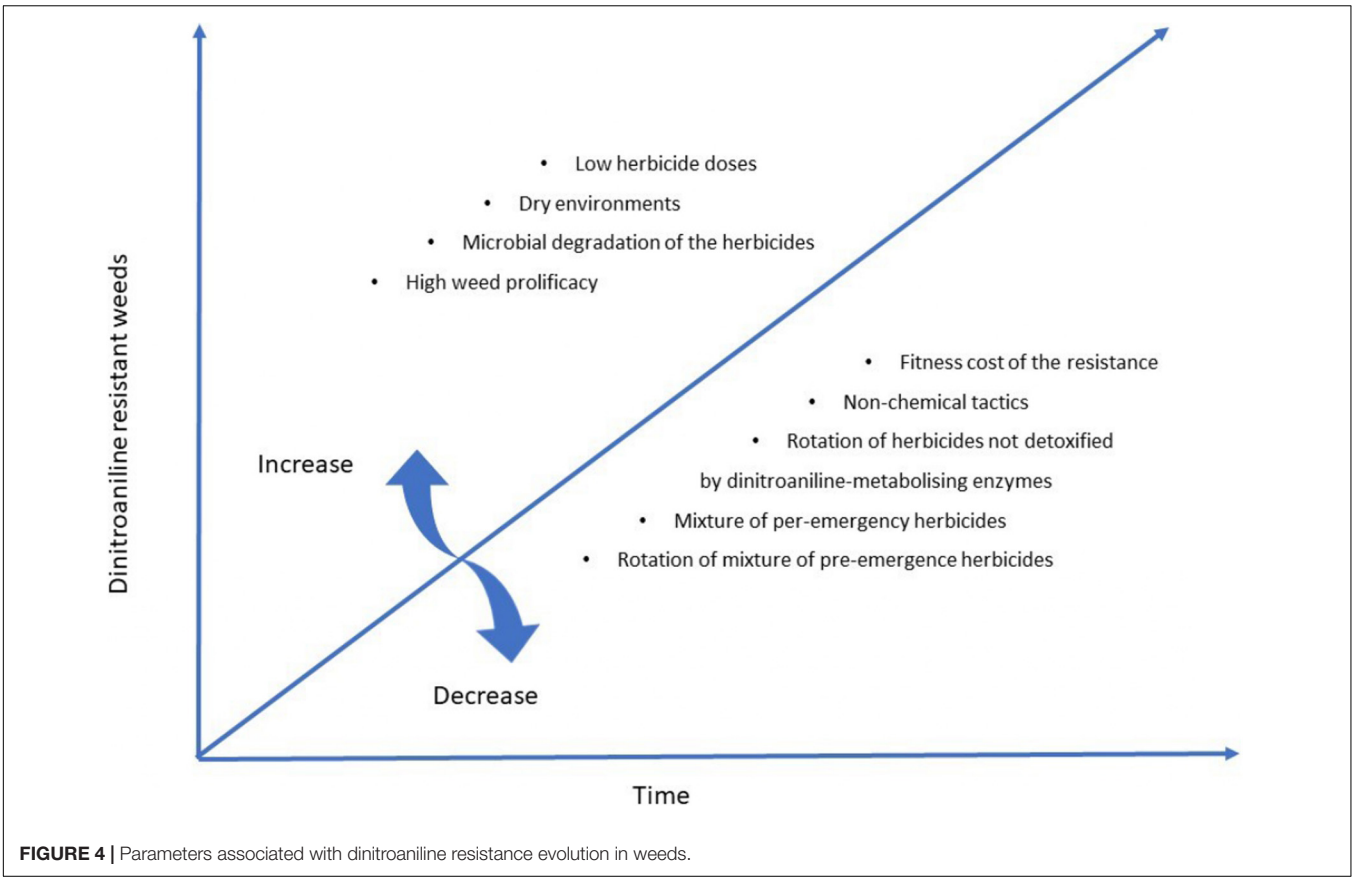

FIGURE 4 | Parameters associated with dinitroaniline resistance evolution in weeds.

mutation and lack of homozygous 243-Met resistant mutant plants in the field.

IMPLICATIONS FOR DINITROANILINE RESISTANCE MANAGEMENT

In terms of resistance management, there are several implications from dinitroaniline-resistance mechanism research. First, given the recessive nature of TSR, dinitroaniline herbicides should be applied at the higher end of the labeled rates to ensure mortality of plants heterozygous for target-site resistance mutations. Secondly, in the case of NTSR, care should be taken when mixing or rotating herbicides to minimize cross-resistance. It would be wise to rotate dinitroaniline herbicides like trifluralin with herbicides that might not be readily metabolized by dinitroaniline-metabolizing enzymes (e.g., prosulfocarb, pyroxasulfone) (Busi et al., 2017, 2020a). Computer simulation modeling, as well as the screening work with many field *L. rigidum* populations showed that mixtures of pre-emergency herbicides (e.g., trifluralin/prosulfocarb, trifluralin/pyroxasulfone, trifluralin/triallate) can delay the onset of resistance and mitigate the existing levels of resistance (Busi and Beckie, 2020; Busi et al., 2020b). Third, co-existence of TSR and NTSR in the same populations suggest the importance of the integrated weed management (IWM) incorporating non-chemical weed management tactics (harvest weed seed control, crop rotation, etc.) to mitigate resistance evolution, and deployment of competitive crop cultivars to suppress dinitroaniline resistant weeds, especially of mutations with concomitant fitness costs. The main message for dinitroaniline herbicide resistance evolution and management is highlighted in **Figure 4**.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Point Mutations as Main Resistance Mechanism Together With P450-Based Metabolism Confer Broad Resistance to Different ALS-Inhibiting Herbicides in *Glebionis coronaria* From Tunisia

Zeineb Hada[1,2], Yosra Menchari[3], Antonia M. Rojano-Delgado[4], Joel Torra[5], Julio Menéndez[6], Candelario Palma-Bautista[4], Rafael de Prado[4] and Thouraya Souissi[1,2]**

[1] Department of Plant Health and Environment, National Institute of Agronomy of Tunisia, University of Carthage, Tunis, Tunisia, [2] Laboratory of Bioagressor and Integrated Management in Agriculture (LR14AGR02), National Institute of Agronomy of Tunisia, University of Carthage, Tunis, Tunisia, [3] Higher Institute of Biotechnology of Beja, University of Jendouba, Jendouba, Tunisia, [4] Department of Agricultural Chemistry and Soil Science, University of Córdoba, Córdoba, Spain, [5] Department d'Hortofructicultura, Botànica i Jardineria, AGROTECNIO-CERCA Center, Universitat de Lleida, Lleida, Spain, [6] Departamento de Ciencias Agroforestales, Escuela Politécnica Superior, Campus Universitario de La Rábida, Huelva, Spain

Resistance to acetolactate synthase (ALS) inhibiting herbicides has recently been reported in *Glebionis coronaria* from wheat fields in northern Tunisia, where the weed is widespread. However, potential resistance mechanisms conferring resistance in these populations are unknown. The aim of this research was to study target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms present in two putative resistant (R) populations. Dose–response experiments, ALS enzyme activity assays, ALS gene sequencing, absorption and translocation experiments with radiolabeled herbicides, and metabolism experiments were carried out for this purpose. Whole plant trials confirmed high resistance levels to tribenuron and cross-resistance to florasulam and imazamox. ALS enzyme activity further confirmed cross-resistance to these three herbicides and also to bispyribac, but not to flucarbazone. Sequence analysis revealed the presence of amino acid substitutions in positions 197, 376, and 574 of the target enzyme. Among the NTSR mechanisms investigated, absorption or translocation did not contribute to resistance, while evidences of the presence of enhanced metabolism were provided. A pretreatment with the cytochrome P450 monooxygenase (P450) inhibitor malathion partially synergized with imazamox in post-emergence but not with tribenuron in dose–response experiments. Additionally, an imazamox hydroxyl metabolite was detected in both R populations in metabolism experiments, which disappeared with the pretreatment with malathion. This study

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***Correspondence:**
Zeineb Hada
zeineb.hada@gmail.com
Thouraya Souissi
souissi.thouraya@iresa.agrinet.tn

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confirms the evolution of cross-resistance to ALS inhibiting herbicides in *G. coronaria* from Tunisia through TSR and NTSR mechanisms. The presence of enhanced metabolism involving P450 is threatening the chemical management of this weed in Tunisian wheat fields, since it might confer cross-resistance to other sites of action.

Keywords: ALS enzymatic activity, florasulam, imazamox, malathion, non-target-site resistance mechanisms, target site resistance mechanism, tribenuron-methyl

INTRODUCTION

Glebionis coronaria (L.) Cass. ex Spach, formerly called *Chrysanthemum coronarium* L., is one of the most troublesome broadleaf annual weeds in cereal crops in northern Tunisia. For years, Tunisian farmers faced challenges managing this weed in wheat fields. It has been estimated that wheat yield losses associated with high densities of *G. coronaria* is up to 75% (Hada et al., 2020). Typically, *G. coronaria* control is still heavily dependent on chemical treatments, mainly acetolactate synthase (ALS)-inhibiting herbicides and synthetic auxin herbicides (SAH). Recently, it has been proven that *G. coronaria* evolved resistance to sulfonylureas (SU), herbicides with potential cross-resistance to triazolopyrimidines – Type 1 (TP) family (Hada et al., 2020). Both herbicide families target the ALS enzyme, which catalyzes the first common step in the biosynthesis of leucine, isoleucine, and valine in plants (Duggleby et al., 2008; Liu et al., 2015). ALS is also the target of three other chemical families, namely, imidazolinones (IMI) (Shaner et al., 1984), sulfonanilides (BTP) (Stidham, 1991), and triazolinones (SCT) (Santel et al., 1999).

The ALS (also referred to as acetohydroxyacid synthase, AHAS) is the target site that is more resistance prone (Yu and Powles, 2014b). So far, evolved resistance to ALS inhibitors has been associated principally to target-site resistance (TSR), by one or more point mutations in the nuclear ALS gene, that disrupt herbicide binding and then reduce the sensitivity of the target enzyme to herbicides (Corbett and Tardif, 2006; Liu et al., 2015). Nowadays, 29 amino acid substitutions at eight different positions, namely, Al122, Pro197, Al205, Asp376, Arg377, Trp574, Ser653, and Gly654, have been documented (Tranel et al., 2019) in 165 weed species around the world (Heap, 2020). These amino acid substitutions result in various cross-resistance patterns among the five chemical families of ALS-inhibiting herbicides depending on weed species (Powles and Yu, 2010; Beckie and Tardif, 2012; Yu and Powles, 2014b; Tranel et al., 2019). For example, resistance to SU has been reported usually as a result of substitution in Pro197 position (Park et al., 2004) and in Gly654 position with less frequency. Amino acid substitutions in Al122, Ala205, Ser653, or Gly654 can endow resistance to IMI but not to SU family (Powles and Yu, 2010; Beckie et al., 2012). Both mutations in Trp574 and Asp376 positions resulted in wide cross-resistance to at least four families of ALS inhibitors (Tranel and Wright, 2002; Powles and Yu, 2010; Tranel et al., 2019).

Recent studies reported the occurrence of polygenic resistance mechanisms that reduce the amount of herbicide reaching ALS target site below lethal levels, known as non-target-site resistance (NTSR) (Deìlye, 2013). In NTSR, there are no significant changes at the ALS target enzyme or ALS enzyme expression level, although this subject is more complicated and less known in both biological and genetic contexts. Metabolic resistance or enhanced rates of herbicide metabolism is one of the major NTSR mechanisms in weeds. Often, it involves major enzymes such as cytochrome P450 monooxygenases (P450s) and glutathione S-transferases (GSTs) that confer to the resistant plant the ability to detoxify herbicides belonging to different chemical classes, and with different modes of action (MoA) (Preston, 2004; Busi et al., 2013, 2014; Yu and Powles, 2014a). ATP-binding cassette (ABC) transporters and enhanced activity of GSTs are also implicated in metabolic herbicide-resistant populations (Yu and Powles, 2014a). Enhanced metabolic resistance to ALS-inhibiting herbicides has been documented principally in grass weeds, such as *Alopecurus myosuroides* Huds. (Moss and Cussans, 1991), *Lolium rigidum* Gaudin (Yu et al., 2009), and *Echinochloa oryzoides* (*Ard.*) *Fritsch* (Yasuor et al., 2009). NTSR to ALS inhibitors have been rarely documented in dicot weeds. Examples are *Sinapis arvensis* L. (Veldhuis et al., 2000), *Amaranthus tuberculatus* (Moq.) Sauer (Guo et al., 2015), and *Papaver rhoeas* L. (Rey-Caballero et al., 2017).

Few cases have been found in which the accumulation of enhanced metabolism and punctual gene mutations coexist and increase resistance levels in weeds. The best-known well-studied case has been a population of *L. rigidum* resistant to chlorsulfuron in Australia (Christopher et al., 1991, 1992). *Rapistrum rugosum* (L.) All. is another weed showing high levels of resistance due to both target gene alteration and enhanced metabolism as resistance mechanisms (Hatami et al., 2016). Coexistence of TSR, such as point mutation, and NTSR (enhanced metabolism) in the same plant has been documented very rarely, particularly in dicots (Rey-Caballero et al., 2017).

This study aimed to investigate the potential TSR and NTSR mechanisms involved in *G. coronaria* resistance to ALS-inhibiting herbicides. The objectives were (1) to evaluate the resistant levels and the potential cross-resistance patterns based on *in vivo* and *in vitro* (ALS enzyme activity) dose–response experiments; (2) to determine if absorption, translocation, and/or enhanced metabolism endow resistance to *G. coronaria*-resistant populations; and (3) to determine the presence of possible point mutations in the ALS gene conferring TSR to *G. coronaria*.

MATERIALS AND METHODS

Plant Materials

For all experiments, three populations of *G. coronaria* were used. Resistant (R) populations (R1 and R2) were selected from a

previous screening of 10 putative R populations collected across the Bizerte region in northern Tunisia, with both populations showing highly resistance levels to SU herbicides (Hada et al., 2020). The susceptible (S) reference population, collected from the roadsides of the National Institute of Agronomy of Tunisia, had never previously received any herbicide treatments. Prior to every experiment, seeds were scarified using sand paper, soaked in 0.3% GA_3 solution for 24 h at room temperature, and then germinated in a growth chamber at 27/17°C day/night and 16 h photoperiod under 350 μmol m^{-2} s^{-1} photosynthetic photon-flux density. Four days after, seedlings with two cotyledons just appearing were transplanted into pots filled with sand and peat mixture (1/3:2/3 *v/v*) and placed in a greenhouse under natural sunlight, 25/15°C, and ∼75% relative humidity, at the Universitat de Lleida and were watered as needed.

Whole-Plant Dose–Response Experiment

The R (R1 and R2) and the S populations were sprayed at 2–4 leaf stage with tribenuron (Granstar 50 SX®, DuPont™, 50 g kg^{-1}), florasulam (Nikos supra®, Dow AgroSciences, 50 g L^{-1}), and imazamox (Pulsar® 40, BASF, 40 g L^{-1}), at increasing doses (**Table 1**). A pretreatment with malathion (organophosphate insecticide, indicator of P450 enzymes involvement in resistance to ALS inhibitors by enhanced metabolism, Christopher et al., 1994) was applied to the plants 1 ½ h prior to both tribenuron and imazamox application. The dose of malathion applied was about 2000 g a.i. ha^{-1}, which was the maximum dose without affecting *G. coronaria* survival or growth, according to preliminary trials (**Supplementary Table 1**). Either herbicides, malathion, or both were applied using a precision bench sprayer with two Hardi ISO LD-110-02 flat fan 110° opening nozzles, operating at a forward speed of 0.9 m s^{-1}, 50 cm above plants, 200 L ha^{-1}, and at a pressure of 215 kPa. Non-treated plants or plants with only malathion were used as controls. The experiment was arranged with six replicates (two plants per pot) per treatment (herbicide with malathion or herbicide without malathion), and per dose. After application, all pots were transferred to the greenhouse and arranged in a completely randomized design. Three weeks after treatment, the percentage of survivals and the fresh weights were recorded. The weight reduction was calculated in respect to corresponding untreated controls for each population and treatment. Experiments were performed twice.

ALS Gene Sequencing

Plants from the S and the R1 and the R2 populations were subject to TSR investigation in a previous unpublished study. DNA was extracted from two susceptible plants from the S population, and four and eight tribenuron-resistant plants from R1 and R2 populations, respectively, using the rapid procedure described by Délye et al. (2015).

Primers GC-ALS 197 (5′-AGGTGGAGCTTCAATGGAGA-3′) and GC-ALS574 (5′-CCTGCAGGAATCATGGGTAA-3′) were used to amplify a 1300-bp ALS fragment of *G. coronaria* corresponding to 433 amino acids. PCR amplifications were performed using KOD FX (Toyobo, Osaka, Japan). Primers were used at a final concentration of 0.3 μM. The cycling program consisted of 94°C for 2 min followed by 35 cycles of 98°C for 10 s, 55°C for 15 s, and 72°C for 2 min. After amplification, PCR products were purified using the ExoSAP-IT PCR Product Clean-UP reagent prior to sequencing.

Crude Enzyme Extraction and ALS Activity

Experiments were conducted to evaluate the ALS enzyme activity in the presence of ALS inhibiting herbicides following the method described by Rojano-Delgado et al. (2015). The ALS activity was measured by means of the quantification of acetoin production (nanomoles of acetoin per milligram of protein per hour). The acetoin product is formed by decarboxylation of acetolactate in the presence of acid. Acetoin reductions indicate that acetolactate production is inhibited due to less ALS enzyme activity during branched-chain amino acid synthesis (Dayan et al., 2015).

The activity of ALS enzyme was conducted in the presence of five ALS inhibitors from different chemical families (bispyribac, florasulam, flucarbazone, imazamox, and tribenuron) using crude extracts isolated from young foliar tissues of the R1, R2, and S populations. For enzyme extraction, 3 g of young leaf tissues were frozen and powdered using liquid nitrogen. Leaf powders were then homogenized in an extraction buffer (in a ratio of 1:3, tissue:buffer), containing 5 g of polyvinylpyrrolidone. The extraction buffer was prepared as a mixture of 0.1 M K-phosphate buffer solution (pH 7.5), 5 mM $MgCl_2$, 10 mM sodium pyruvate, 100 μM flavin adenine dinucleotide, 50 mM thiamine pyrophosphate, 12 mM dithiothreitol, and glycerol–water (1:9, *v/v*). This homogenate was agitated for 10 min at 4°C, filtered through four layers of cheesecloth, and centrifuged for 20 min (20,000 rpm) to separate the supernatant. The supernatant obtained was immediately used for ALS enzyme activity assays.

Acetolactate synthase activity was assayed by adding 0.05 ml of enzyme extract to 0.1 ml of freshly prepared assay buffer [0.08 M potassium phosphate (KH_2PO_4/K_2HPO_4), pH 7.5, 0.15 M sodium pyruvate, 1.5 mM $MgCl_2$, and 1000 μM FAD] and increasing concentrations of herbicides (0–1000 μM). After mixture and incubation (37°C for 1 h), the reaction was stopped by the addition of 0.05 ml of H_2SO_4 (3 M). The reaction tubes were then heated (15 min at 60°C) to facilitate decarboxylation of acetolactate to acetoin. Acetoin was detected as a colored complex (520 nm) formed after the addition of 0.25 ml of creatine (5 g L^{-1}, freshly prepared in water) and 0.25 ml of α-naphthol (50 g L^{-1}, freshly prepared in 5 M NaOH) and then incubated (60°C for 15 min). Background was determined using control vials in which the reaction was stopped before the incubation and subtracted. Maximum specific activity of ALS was measured in the absence of herbicides. The experiment was performed with three replications per herbicide concentration and per population, and repeated twice.

Absorption and Translocation of ^{14}C-Tribenuron Experiment

Radiolabeled herbicide solution was prepared by mixing commercial tribenuron herbicide at the recommended field dose

TABLE 1 | Herbicide range of doses applied to resistant (R) and susceptible (S) populations with or without malathion.

Herbicide	Field rate (g a.i. ha^{-1})	Malathion*	Pop	Rates (g a.i. ha^{-1})						
Tribenuron	18.7	±	R	18.75	37.5	56.25	75	150	300	0
		±	S	0.29	0.59	1.17	2.34	4.69	9.38	0
Florasulam	7.5	−	R	3.75	7.5	15	30	60	0	
		−	S	0.47	0.94	1.88	3.75	7.50	0	
Imazamox	50	±	R	25	50	75	100	200	0	
		±	S	1.56	3.13	6.25	12.5	25	50	

** (±) means that both treatments with malathion and without malathion were applied; (−) means that only herbicide without malathion treatment was applied.*

(18.7 g a.i. ha^{-1}), with labeled herbicide (^{14}C-tribenuron, specific activity of 1.422 MBq $mmol^{-1}$, Institute of Isotopes Co., Ltd., Budapest, Hungary). At the two-leaf stage, plants of the R and the S populations received on one of the two leaf surfaces four 0.5-µl droplets of herbicide mixture using a microapplicator (Hamilton PB 6000 dispenser, Hamilton, Co., Reno, NV, United States). Every plant received a total radioactivity of 0.67 KBq μl^{-1}. Five plants per population and per sampling time (expressed as hours after treatment, HAT) were considered as repetitions. In each sampling time (12, 24, 48, and 72 HAT), plants were removed from pots, roots were carefully washed with distilled water, and treated leaf (TL) was separated from the rest of aerial section (AS) and root section (RS). Unabsorbed ^{14}C-tribenuron was washed from every TL using 2 ml of acetone–water solution in a ratio of 1:1 (*v/v*), the washes were mixed with 15 ml of scintillation fluid (Ultima Gold^{TM}, Perkin-Elmer, Packard Bioscience BV), and radioactivity was quantified by liquid scintillation spectrometry (LSS, 6000 TA scintillation counter, Beckman Instruments, CA, United States). The plant sections were dried for 48 h at 70°C, and combusted in a biological sample oxidizer (OX 500; R. J. Harvey Instrument, Tappan, NY, United States). The released $^{14}CO_2$ was trapped in 18 ml of Oxysolve C400 (Zinsser Analytic, Frankfurt), and its associated radioactivity was determined by LSS.

The translocation of ^{14}C-tribenuron in each plant section was determined and expressed as a percentage of total absorbed radioactivity. The average total recovery of the applied ^{14}C-tribenuron was greater than 88%, both in S and R populations. For qualitative study of radiolabeled tribenuron, three plants per population were removed from the soil at 24, 48, and 72 HAT. Roots were rinsed and whole plants were pressed and dried at room temperature for 4 days. Then, plants were pressed against a phosphor storage film (25 cm × 2.5 cm, PerkinElmer Life and Analytical Sciences, Shelton, CT, United States), for 6 h. Dried plants were scanned using a phosphor imager Cyclone (Perkin-Elmer, Packard Bioscience BV).

The absorption and the translocation were calculated following these two expressions:

$$\text{Absorption (\%)} = \frac{\text{the radioactivity recovered from plant section}}{\text{total radioactivity recovered}} \times 100$$

$$\text{Translocation (\%)} = \frac{\text{the absorbed radioactivity in TL, AS, or RS}}{\text{absorbed radioactivity in all plant sections}} \times 100$$

Enhanced Metabolism Study of Imazamox Herbicide

Enhanced metabolism in *G. coronaria* populations was investigated by applying, at field rate, imazamox herbicide (50 g a.i. ha^{-1}) alone and imazamox plus a pretreatment of malathion (2000 g a.i. ha^{-1}). Two groups of controls were used: one group of non-treated plants and the second group of plants treated only with malathion. Plants were harvested at different times (0, 48, 72, and 96 HAT). Prior to the extraction, each plant was washed with 60 ml of water to remove traces of imazamox and soil on the leaf surface. The washed plants were divided into roots and shoots. The extraction and detection of metabolites were previously described by Rojano-Delgado et al. (2014). One-half gram of each plant sample was ground to powder in liquid nitrogen and mixed with a methanol–water solution (10 ml, 90:10 *v/v*), the mixture was ultra-sonicated during 10 min (at 70 W, duty cycle 0.7 s s^{-1}) and then centrifuged for 15 min (at 20,000 rpm). Six milliliters of supernatant was collected and evaporated under an air stream, and later, 0.5 ml of extractant (methanol–water, 90:10 *v/v*) was added to reconstitute the sample. The new solution was filtered through a 45-µm pore-filter syringe (13 mm i.d. from Millipore, Carrigtwohill, Ireland) and used for liquid chromatographic quantification. Fifty microliters of the reconstituted solution was injected into a liquid chromatography system. A Gold HPLC (high-performance liquid chromatography) System (Beckman Coulter, Fullerton, CA, United States), equipped with a DAD (wavelength range 190–600 nm), was used to detect different analytes. The imazamox and metabolite separation was performed by using a hydrophilic interaction liquid chromatography column (HILIC; 3 µm particle size, 20 cm × 4.6 cm, Phenomenex, California, CA, United States), with a constant flow rate of 1.0 ml min^{-1} at 40°C. In this experiment, the wavelength of 240 nm was measured. In mobile phase A, 1% of acetic acid–water (*v/v*) was used, and in mobile phase B, pure methanol was used. The gradient elution program begun with first 5% mobile phase B and then linear gradient composed of (i) 5–20% methanol in 10 min; (ii) 20–80% methanol in 10 min; (iii) 80–100% methanol in 5 min; and (iv) 100–5% methanol in 10 min.

Target compounds were detected based on the retention times referring to the imazamox standard. The metabolites detected were quantified based on the calibration curve of imazamox, and results were given as concentrations ($\mu g\ g^{-1}$) of imazamox and metabolites. The experiment was performed for

each population using three replicates per sampling time, and two repetitions.

Statistical Analysis

One-way ANOVA was performed using the SPSS-20 software (IBM, NY, United States) to analyze the dose–response data, for both whole-plant and ALS activity experiments, and means were compared using the Duncan *post hoc* pairwise test (*P*-value = 0.05). Four-parameter log-logistic regression models (Eq. 1) were fitted to determine the herbicide concentrations required to decrease the ALS activity (I_{50}), fresh weight (ED_{50}), and survival (LD_{50}) by 50% in the three populations. The fitting for the dose–response experiment was performed using Sigmaplot 11.0 (Systat Software, San Jose, CA, United States), and the fitting for ALS enzyme activity was performed using R software (drc package). The resistance index (RI) of the R1 and the R2 populations to different ALS inhibitors tested were calculated as $RI = LD_{50}$ (R)/LD_{50} (S), $RI = ED_{50}$ (R)/ED_{50} (S), or $RI = I_{50}$ (R)/I_{50} (S).

$$y = c + \frac{(D - C)}{1 + \left(\frac{x}{x50}\right)^{-b}} \quad (1)$$

c, the lower limit adjusted to 0; d, the upper limit adjusted to 100; b, Hill's slope at x_{50} (inflection point, representing the effective herbicide dose required for 50% reduction in ALS activity, fresh weights, and survivals); x was the independent variable [dose of herbicide applied (g a.i. ha^{-1}], and y was the dependent variable (ALS activity, fresh weight, or survival) expressed as a percentage of the untreated control.

For the ^{14}C-tribenuron experiment, a two-way ANOVA was conducted to determine the effect of populations, sampling time, as well as their interaction on herbicide absorption and translocation. Data from the imazamox metabolism experiment

TABLE 2 | Parameters of the log-logistic equation of the dose–response regression curves of survival and fresh weight in *G. coronaria* S and R (R1 and R2) populations in presence and absence of malathion.

	Herbicide	Malathion	Population	LD_{50}/ED_{50} (g a.i./ha)[a]	Slope	RI
% Survivals	Tribenuron	+	S	1.7	−1.7	–
			R1	>300	–	>300
			R2	>300	–	>300
		−	S	1.7	−2.4	–
			R1	>300	–	>300
			R2	>300	–	>300
	Imazamox	+	S	8.0	−1.8	–
			R1	49.5	−3.0	6.2*
			R2	49.9	−2.8	6.2**
		−	S	8.3	−3.0	–
			R1	63.8	−3.4	7.9**
			R2	69.7	−3.1	8.7*
	Florasulam	−	S	0.6	−0.9	–
			R1	5.2	−0.9	8.4***
			R2	10.2	−1.7	16.5***
% Fresh weight	Tribenuron	+	S	0.8	1.8	–
			R1	>300	–	>300
			R2	>300	–	>300
		−	S	1.4	2.7	–
			R1	>300	–	>300
			R2	>300	–	>300
	Imazamox	+	S	6.5	2.2	–
			R1	20.8	1.6	3.19***
			R2	24.8	1.7	3.81***
		−	S	5.6	2.4	–
			R1	27.7	1.6	4.25***
			R2	21.1	1.4	3.24***
	Florasulam	−	S	0.5	1.3	–
			R1	2.2	0.9	4.67***
			R2	4.0	1.1	8.66***

RI, resistance index.

**P = 0.05, **P = 0.01, ***P = 0.001.*

[a] *LD_{50}/ED_{50} represent the herbicide concentrations required to decrease by 50% the survival (%) and fresh weight (%) of G. coronaria, respectively.*

was subjected to one-way ANOVA. Means were compared using Duncan *post hoc* pairwise test (P-value = 0.05).

RESULTS

Cross-Resistance Pattern and Malathion Effect on Resistance

Both R1 and R2 populations exhibited resistance in response to tribenuron, imazamox, and florasulam based on survival and fresh weight reduction (%) recorded in this study. The highest resistance level (greater than 300) was attributed to tribenuron herbicide. In tribenuron-treated R1 and R2 plants, increasing rates of herbicide did not significantly affect the survival and fresh weight reduction (%), as the ED_{50} and LD_{50} values exceeded the highest applied dose (300 g a.i. ha^{-1}). Thus, both R populations survived up to 16-fold the recommended field dose (**Table 2**).

Regarding florasulam resistance, the R2 population was two times more resistant than the R1, showing higher survival rates and less fresh weight reduction (LD_{50} = 10.2 g a.i. ha^{-1} and ED_{50} = 4.0 g a.i. ha^{-1} respectively, **Table 2** and **Figure 1**). Lower resistance levels were attributed to imazamox herbicides as presented by the RI (**Table 2**). The two R populations of *G. coronaria* showed similar survival rates with higher LD_{50} in R2 compared to the R1 plants (69.7 and 63.8 g a.i. ha^{-1}, respectively). In contrast, fresh weight reduction was slightly higher in R1; ED_{50} was about 27.7 and 21.1 for the R1 and the R2, respectively.

A pretreatment with malathion partially increased the sensitivity of both *G. coronaria* R populations to imazamox (**Figure 1** and **Table 2**). This was clearer in survival data, as both R1 and R2 exhibited 6.2 more resistance than the S plants, showing 21 and 29% reduction of resistance levels compared to plants treated only with imazamox. The results of fresh weight measurements supported these findings only for the R1 population (**Table 2**), together with the visual inspection of plant sizes (**Supplementary Figure 1**). Finally, malathion had no effect on tribenuron resistance *in G. coronaria*. These findings supported the hypothesis that enhanced imazamox metabolism may be present at least in the R1 population.

ALS Activity

In the absence of ALS inhibitors, the R1, R2, and S populations presented similar activity of extracted ALS. The basal activities were 305.9 (±18.6), 321.1 (±9.4), and 317.4 (±11.9) nmol acetoin mg TSP^{-1} h^{-1}, respectively. However, results revealed significant differences in ALS activity between the R and the S populations when exposed to ALS-inhibiting herbicides (**Table 3** and **Figure 2**). The I_{50} values ranged from 6.81 e^{-02} to 3.34 μM

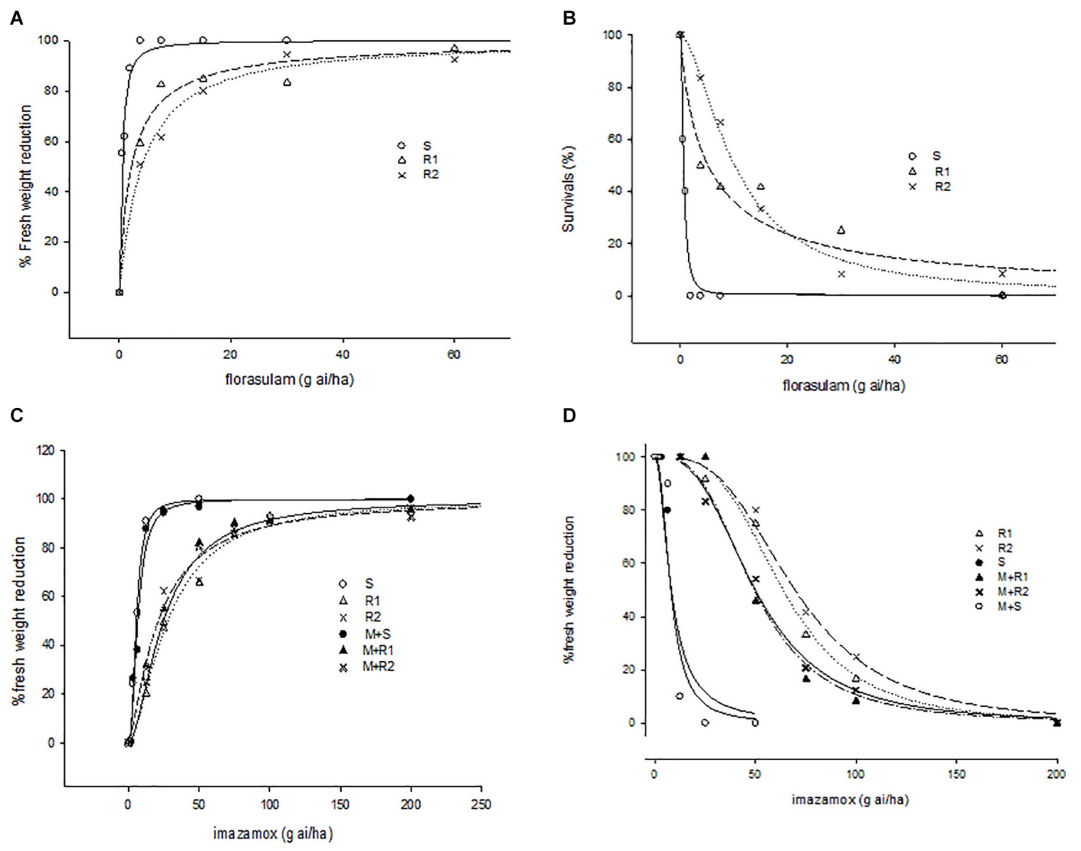

FIGURE 1 | Response of fresh weight and survivals of susceptible and resistant populations of *G. coronaria* to different concentrations of florasulam **(A,B)** and imazamox **(C,D)**.

TABLE 3 | The resistance levels of different *G. coronaria* populations to bispyribac, florasulam, flucarbazone, imazamox, and tribenuron herbicides.

Herbicides (chemical family)	Population	I_{50} (μM)	*F*-value	RI
Bispyribac (BTP)	S	3.34		–
	R1	33.80	48.945***	10.242
	R2	45.20		13.532
Florasulam (TP)	S	1.15 e^{-02}		–
	R1	3.76 e^{-02}	11.732***	3.270
	R2	6.34 e^{-02}		5.513
Flucarbazone (SCT)	S	6.81 e^{-02}		–
	R1	1.17 e^{-01}	11.645***	1.718
	R2	1.15 e^{-01}		1.689
Imazamox (IMI)	S	1.22		–
	R1	12.03	4.6486***	9.861
	R2	6.68		5.475
Tribenuron (SU)	S	0.44		–
	R1	16.02	2.2309***	36.409
	R2	11.91		27.068

*SU, sulfonylurea; IMI, imidazolinone; TP, triazolopyrimidine; PTB, pyrimidinyl-thiobenzoates; SCT, sulfonyl-aminocarbonyl-triazolinone; I_{50}, herbicide dose required to inhibit ALS activity by 50% compared with that of the untreated control, R1 and R2, resistant population; S, susceptible population. RI, resistance index = I_{50} (R1)/I_{50} (S); (RI) = I_{50} (R2)/I_{50}. ***: the differences between populations are highly significant according to the Duncan test (P = 0.001).*

for the S population, 3.76 e^{-02} to 33.80 μM for R1, and 6.34 e^{-02} to 45.20 μM for R2.

Higher values of RI in tribenuron and bispyribac were found. Resistant plants of the R1 and the R2 required a tribenuron concentration of 36 and 27 times higher respectively than the S plants for similar inhibition of ALS activity. The I_{50} values were only 1.7-fold higher in both R populations compared with the S population treated with flucarbazone, indicating almost no resistance to this herbicide. Results also showed that resistance to tested herbicides varied among the two populations R1 and R2. The quantification of the ALS activity revealed that the R1 was more resistant to tribenuron and imazamox, while the R2 was more resistant to bispyribac and florasulam. Based on the RI estimated by the ALS activity assays, resistance to ALS inhibitors may be classified as follows: flucarbazone < florasulam < imazamox < bispyribac < tribenuron. Furthermore, our results might suggest that both R1 and R2 populations developed cross-resistance to four out of the five chemical families of the ALS-inhibiting herbicides used in this study.

ALS Sequencing

Comparison of ALS gene sequences in susceptible (accession number MW598184) and resistant plants (accession numbers MW598185 to MW598189) revealed three non-synonymous mutations at positions 197, 376, and 574 standardized to the ALS protein sequence of *Arabidopsis thaliana* (**Supplementary Figure 2**), which are already known to be involved in sensitivity to ALS-inhibiting herbicides in weeds (Tranel et al., 2019). At codon 197, the amino acid substitution Pro (CCA) to Thr (ACA) was detected in susceptible and resistant plants. However, the Arg197, Ser197, and Gln197 substitutions caused by variable nucleotide positions were only detected in the R2 population. The amino acid change at position 376 was caused by a substitution from Asp (GAT) to Glu (GAA) in three plants from the R2 population. A point mutation at the second base of the amino acid Trp (TGG), which resulted in the substitution by Leu (TTG), was found in one homozygous and one heterozygous plant from the R1 population (**Table 4**). These findings showed that most R plants sequenced contained at least one mutant-resistant allele, suggesting that the TSR mechanism is relevant in *G. coronaria* resistance to ALS-inhibiting herbicides.

Absorption and Translocation

Statistical analysis showed no significant differences ($P > 0.05$) in absorption patterns between populations, as the penetration of labeled herbicide remained asymptotic. Overall, the ^{14}C−tribenuron absorption was less than 30% for all populations. No significant differences were found in absorption percentages between R and S populations over time, although the R2 population was able to absorb more labeled herbicide (27%) at the end of the experiment, compared to the R1 and the S populations (22 and 24%) (**Table 5**).

According to the translocation data (**Table 5**), the highest amount of the labeled herbicide remained in the treated leaves, representing more than 90% of the recovered radioactivity at 72 HAT. The lowest amounts of radioactivity were detected in both shoots and roots in a similar distribution pattern for all populations. However, significant differences in herbicide translocation were found between populations until 24 HAT. Only 86% of radioactivity remained in the R2-treated leaves compared to 92 and 93% in the S and the R1 plants, respectively. Similarly, 13% of radioactivity was found in the aerial part of the R2 plants, while only 7 and 5% of radioactivity were detected in the S and the R1, respectively. These differences remained until 24 HAT but faded over time, indicating a similar pattern of tribenuron herbicide translocation in all tested populations.

Thus, neither absorption nor translocation mechanisms could be responsible for the high levels of tribenuron resistance observed in both R1 and R2 populations. Phosphor images illustrated a similar level of ^{14}C−tribenuron movement in the R and the S populations, showing minimal translocation to the rest of the AS and the roots (**Figure 3**).

Imazamox Metabolism Studies

In all sampling times, higher amounts of herbicide were detected in the whole plants (foliar and roots) of the S population, especially at 72 and 96 HAT, compared to the R1- and the R2-resistant populations (**Table 6**). In the absence of malathion, the amount of imazamox was significantly different among populations. However, it was statistically similar in leaves when plants were pretreated with malathion. Chromatographic analysis permitted the detection of a hydroxyl metabolite of imazamox (imazamox-OH) at 20 min (**Supplementary Figure 2**). No imazamox metabolite was formed in the leaves and roots of plants pretreated with malathion in all tested populations.

In the absence of malathion pretreatment, the imazamox-OH was detected at all sampling times for the R1 population and only at 96 HAT for the R2 population. Additionally,

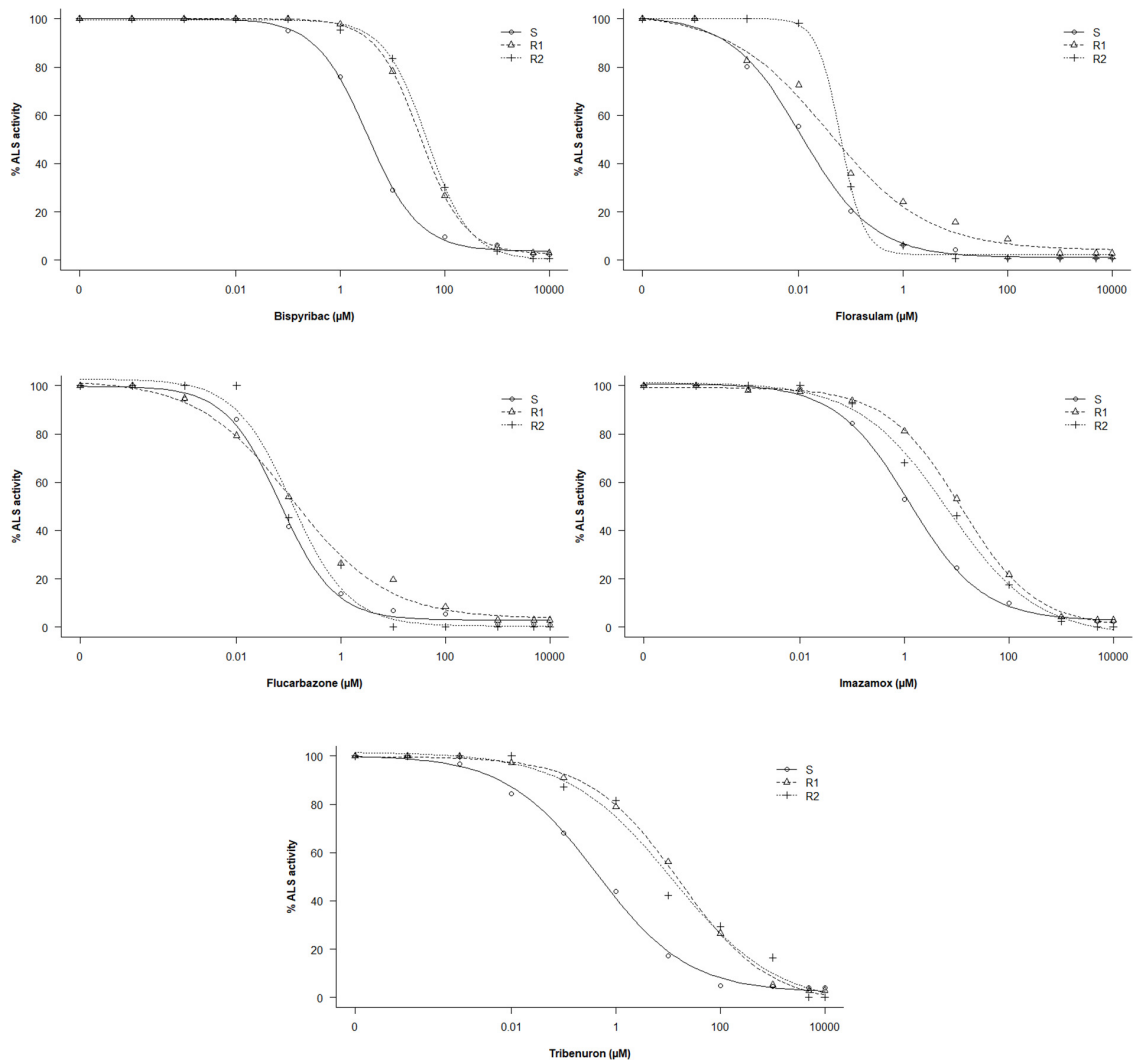

FIGURE 2 | Dose–response curves of the ALS enzyme activity of *G. coronaria* populations exposed to increased concentrations (µM) of five herbicides.

imazamox metabolites were not detected in the S plants over time (**Supplementary Figure 3**), indicating different metabolism patterns between the R and the S populations. These results may suggest the important role played by a P450 enzyme system, inhibited by the pretreatment with malathion, conferring greater resistance through the hydroxylation of imazamox (first step in metabolism) in the R1 and the R2 plants when exposed to the herbicide. Statistical analysis showed significant differences in imazamox and imazamox-OH concentrations between the R1 and R2 populations. At 48 HAT and in the absence of malathion, the amount of imazamox in the R1 population was significantly lower than in the R2. Imazamox herbicide was detected in the roots of the R1 population while it was found only in foliar part of the R2. The imazamox-OH was first detected in leaves of the R1 population and then in both leaves and roots at the concentrations of 7.9 $\mu g\ g^{-1}$ and 2.8 $\mu g\ g^{-1}$, respectively. No metabolite was detected in the R2 population until 96 HAT. The detection of imazamox-OH in the R2 population occurred at a very low concentration (1.6 $\mu g\ g^{-1}$), compared to the one detected in the R1 (8.9 $\mu g\ g^{-1}$).

DISCUSSION

Results from this study revealed the development of cross-resistance to ALS-inhibiting herbicides in two *G. coronaria* populations from the Bizerte region in northern Tunisia. Since their introduction in 1999, the SU herbicides were excessively used by the Tunisian wheat growers to control troublesome grass weeds such as *L. rigidum* (Khammassi et al., 2020) and dicotyledonous weeds. The main reason was their broad-spectrum weed control at low rates. A previous survey, conducted in the same region, showed that florasulam was frequently used in a mixture with SAH to control *G. coronaria* in cereal crops (Hada et al., 2020) while imazamox has never been applied on the weed to our knowledge.

TABLE 4 | Different amino acid substitutions detected at codons Pro197, Asp376, and Trp574 of the ALS gene from S (susceptible) and R1 and R2 populations (resistant) of *G. coronaria* based on amino acid positions in *Arabidopsis thaliana.*

Population	Plant	Substitutions*		
		Pro197CCA	Asp376GAT	Trp574TGG
S	1	Pro/ThrCCA/**A**CA*	–	–
	2	Pro/ThrCCA/**A**CA	–	–
R1	1	–	–	Leu/LeuT**T**G/T**T**G
	2	–	–	Trp/LeuTGG/T**T**G
	3	Thr/Thr**ACA/A**CA	–	–
	4	Pro/Gln**A**CA/**CA**A	–	–
R2	1	Pro/SerCCA/**T**CA	–	–
	2	Thr/Ser**A**CA/**T**CA	–	–
	3	Gln/GlnC**A**A/C**A**A	–	–
	4	Gln/GlnC**AA**/C**A**A	–	–
	5	Pro/ArgCCA/C**G**A	Asp/GluGAT/GA**A**	–
	6	–	Asp/GluGAT/GA**A**	–
	7	–	Asp/GluGAT/GA**A**	–
	8	–	Asp/GluGAT/GA**A**	–

**Nucleotide changes are in bold.*

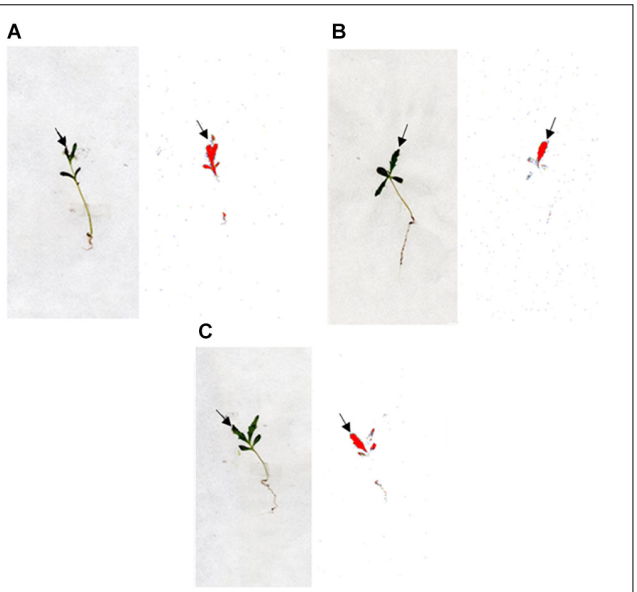

FIGURE 3 | Digital photographs (plants on the left) and autoradiographic photographs of the phosphor imager (plants on the right) showing the distribution of ^{14}C-tribenuron within *G. coronaria* plants of population S, R1, and R2 (**A**, **B**, and **C**, respectively) at 72 HAT. The highest concentration of ^{14}C is highlighted in red.

Data on the survival and the fresh weight reduction showed that both R1 and R2 populations exhibited high resistance levels to tribenuron and were less resistant to florasulam and imazamox. Similar results were found in *P. rhoeas* showing that the degree of resistance varied among ALS inhibitors, with RI lowest for florasulam and imazamox than for tribenuron (Kaloumenos et al., 2011). Our findings are in line with the only known study conducted by Tal and Rubin (2004) on the occurrence of resistance to ALS-inhibiting herbicides in *G. coronaria* populations, showing high RI (greater than 729) to

TABLE 5 | Absorption (expressed as % of recovered radioactivity) and translocation to different plant organs (expressed as % of absorbed radioactivity) of ^{14}C-tribenuron in S, R1, and R2 populations of *G. coronaria* at 12, 24, 48, and 72 HAT.

Population	HAT	Absorption (%)	Translocation (%)		
			Treated leaf (TL)	Rest of aerial section (AS)	Root section (RS)
S	12	24.0 ± 4.1	92.2 ± 1.9	7.3 ± 1.9	0.5 ± 0.1
	24	29.6 ± 9.6	96.1 ± 0.3	3.5 ± 0.3	0.4 ± 0.2
	48	23.2 ± 3.6	89.7 ± 2.6	9.9 ± 2.6	0.4 ± 0.1
	72	23.8 ± 1.4	91.4 ± 2.3	8.2 ± 2.3	0.4 ± 0.0
R1	12	20.6 ± 1.5	93.6 ± 2.3	5.8 ± 2.2	0.5 ± 0.0
	24	22.9 ± 2.0	90.9 ± 3.2	8.6 ± 3.2	0.4 ± 0.0
	48	22.9 ± 3.6	92.6 ± 2.0	7.1 ± 2.0	0.4 ± 0.0
	72	21.8 ± 1.5	93.2 ± 2.4	6.4 ± 2.4	0.4 ± 0.0
R2	12	22.0 ± 2.9	86.5 ± 1.3	13.0 ± 1.2	0.5 ± 0.1
	24	21.0 ± 1.9	88.2 ± 1.4	11.1 ± 1.5	0.7 ± 0.3
	48	22.0 ± 3.4	90.5 ± 0.4	9.0 ± 0.5	0.5 ± 0.1
	72	27.4 ± 2.5	90.9 ± 2.1	8.8 ± 2.0	0.3 ± 0.0

Means of five repetitions per biotype ± values of standard errors are given.

ANOVA	Absorption (%)	Treated leaf (TL)	Rest of aerial section (AS)	Root section (RS)
Population	$P = 0.331^{ns}$	$P = 0.016^{**}$	$P = 0.019^{**}$	$P = 0.459^{ns}$
HAT	$P = 0.712^{ns}$	$P = 0.856^{ns}$	$P = 0.873$ ns	$P = 0.258^{ns}$
Population × HAT	$P = 0.542^{ns}$	$P = 0.134^{ns}$	$P = 0.142$ ns	$P = 0.497^{ns}$

*^{ns}No significant differences were found; **significant differences were found at P = 0.05 based on Duncan test.*

TABLE 6 | Imazamox concentration and its hydroxyl metabolite (Imaza-OH) in foliar and root extracts from R (R1 and R2) and S populations of *G. coronaria* at 48, 72, and 96 HAT ($\mu g/g^{-1}$).

HAT	Pop	−Malathion				+ Malathion			
		Imazamox		Imaza-OH		Imazamox		Imaza-OH	
		Foliar	Root	Foliar	Root	Foliar	Root	Foliar	Root
48	S	32.4 ± 2.1b	9.9 ± 0.4b	ND	ND	30.2 ± 2.1a	12.7 ± 19b	ND	ND
	R1	25.2 ± 1.9a	1.7 ± 0.4a	5.9 ± 0.2	ND	29.6 ± 0.5a	9.7 ± 0.4ab	ND	ND
	R2	33.0 ± 2.4b	ND	ND	ND	29.2 ± 0.9a	7.0 ± 1.3a	ND	ND
	F-value	8.07*	132.5***	–	–	0.3^{ns}	8.8*	–	–
72	S	36.0 ± 2.8a	25.5 ± 2.7c	ND	ND	39.3 ± 0.8a	31.9 ± 3.1b	ND	ND
	R1	48.1 ± 2.2b	2.9 ± 0.5b	7.9 ± 0.4	2.8 ± 0.5	38.0 ± 0.6a	26.9 ± 0.5b	ND	ND
	R2	48.2 ± 1.6b	2.7 ± 0.3a	ND	ND	36.4 ± 2.4a	15.7 ± 2.1a	ND	ND
	F-value	19.6**	112***	–	–	1.9^{ns}	29.3***	–	–
96	S	31.3 ± 3.9a	42.5 ± 1.7c	ND	ND	38.5 ± 1.5a	50.0 ± 1.3c	ND	ND
	R1	42.5 ± 4.0b	8.8 ± 0.b	8.9 ± 0.3b	9.9 ± 1.6	36.6 ± 1.1a	38.9 ± 0.2b	ND	ND
	R2	63.3 ± 3.0c	4.0 ± 0.3a	1.6 ± 0.4a	ND	36.9 ± 1.1a	32.0 ± 1.2a	ND	ND
	F-value	43.2***	858.4***	732.1***	–	1.4^{ns}	181.1***	–	–

*$P = 0.05$, **$P = 0.01$, ***$P = 0.001$

[a,b,c]*Different letters denote significantly differences between populations in imazamox concentration and its hydroxyl metabolite (Imaza-OH) in foliar and root extracts at different sampling time according to the Duncan test ($p = 0.05$).*

tribenuron and cross-resistance to all chemical families of ALS-inhibiting herbicides (RI ranging between 4 and 48). There is an abundant literature demonstrating resistance to ALS-inhibiting herbicides in dicotyledonous weed species including *Salix alba* in Spain (Rosario et al., 2011), *Myosoton aquaticum* (L.) Moench in China (Liu et al., 2015), *R. rugosum* in Iran (Hatami et al., 2016), *P. rhoeas* in Spain (Rey-Caballero et al., 2017), and *Amaranthus palmeri* in the United States (Küpper et al., 2017). It is common for these species to develop resistance to herbicides belonging to SU chemical family, as well as cross-resistance to herbicides from other families within ALS inhibitors.

The results of *in vitro* ALS activity further confirmed the cross-resistance to florasulam, imazamox, tribenuron, and also to bispyribac. A lower RI (<2) was determined for flucarbazone. Gene sequencing revealed amino acid replacements in positions Pro197, Asp376, and Trp574. Both mutations in Trp574 and Asp376 positions resulted in wide cross-resistance to at least four families of ALS inhibitors (Tranel and Wright, 2002; Powles and Yu, 2010; Tranel et al., 2019). Amino acid substitutions in Pro197 were previously reported in resistant *G. coronaria* (Tal and Rubin, 2004). These authors found that Thr197 and Ser197 substitutions conferred high resistance levels to tribenuron. However, in this study, the Thr197 substitution was also detected in susceptible plants. In *Monochoria korsakowii*, a Pro197Leu codon known to confer resistance in other species was detected in a pseudogene (Iwakami et al., 2020). These point mutations are widely reported in other dicotyledonous weeds as endowing resistance to SU (Cruz-Hipolito et al., 2013; Yu and Powles, 2014a; Liu et al., 2015) and cross-resistance to other chemical families (Hatami et al., 2016). Our findings, which are in line with the previous studies, suggest that the cross-resistance pattern found in this study may be explained by the substitutions in Pro197, Asp376, and Trp574 and that target-site alteration may be the dominant mechanism of resistance developed by *G. coronaria* to ALS-inhibiting herbicides. However, it is important to determine the copy number of ALS genes and their transcription in *G. coronaria* to better understand the evolution of target-site herbicide resistance. Despite the fact that SU and TP herbicide families are the only ones applied by Tunisian farmers in wheat fields, the weed has developed resistance to other ALS inhibitor families that have never been used before, which might hamper the use of any new ALS inhibitor in the cereal fields in this northern region of Tunisia.

The levels of tribenuron absorption and translocation in the R and the S populations were also investigated in this study. Based on the results, neither absorption nor translocation contributed as mechanisms to *G. coronaria* resistance to ALS-inhibiting herbicides. Indeed, both mechanisms rarely underlay resistance to the ALS inhibitors (Veldhuis et al., 2000; Cruz-Hipolito et al., 2013; Riar et al., 2013; Yu and Powles, 2014b). These results further support the reports from other studies on *Conyza sumatrensis* (Retz.) E. Walker (Osuna and De Prado, 2003), *S. alba* (Rosario et al., 2011), or *R. rugosum* (Hatami et al., 2016). However, several resistant weed species such as *P. rhoeas* (Rey-Caballero et al., 2017) were able to translocate more herbicide than the susceptible ones, suggesting that ALS inhibitors may affect the transport of assimilates into the phloem. Previous work on *Pisum sativum* L. reported that carbohydrates were excessively accumulated in leaves of S plants after herbicide application (Zabalza et al., 2004). Similar results were also reported on *Thlaspi arvense* L. (Bestman et al., 1990).

In dose–response experiments, pretreatment with the P450 inhibitor malathion had no effect on the efficacy of tribenuron. However, it partially synergized with imazamox, leading to a shift toward sensitivity in both R populations. This indicates that P450 would be involved in the resistance response of *G. coronaria* to ALS-inhibiting herbicides, at least those of the IMI family. The P450 superfamily is the largest enzymatic protein family in plants, linked to vital functions, participating in the

synthesis of fatty acids, sterols, and hormones. Members of this superfamily are involved in multiple metabolic pathways with distinct and complex functions, mediating a vast array of reactions (Jun et al., 2015). Furthermore, it is known that this family of enzymes is responsible for the detoxification processes of xenobiotics, in particular of herbicides in plants (Powles and Yu, 2010). Malathion has an inhibiting effect on P450 in plants, which can no longer catalyze herbicide degradation. Therefore, R plants may totally or partially, as in the case of this study, lose their resistance.

Enhanced metabolism, likely meditated by P450 in both R1 and R2 populations, was confirmed by HPLC experiments. This is the first report of the presence of NTSR mechanisms in *G. coronaria*. Indeed, imazamox-hydroxyl metabolite was detected in both R1 and R2 populations and disappeared with the pretreatment with malathion. Comparing both R populations, the R1 was able to detoxify imazamox faster than the R2 did. In fact, the faster an herbicide is metabolized, the less it is available for translocation and activity at the site of action (Zimdahl, 2007). This may explain the differences in the translocated amounts of imazamox between the two R populations. Previous works in *L. rigidum* (Busi et al., 2011) and *A. myosuroides* (Petit et al., 2011) pointed out that metabolic resistance can be controlled by polygenic loci and that these loci were involved in NTSR to ALS inhibitors depending on plants and populations. Recent studies characterized P450-based metabolic cross-resistant individuals in *L. rigidum* and hypothesized that the genetic control was probably of polygenic and quantitative nature at the population level (Yu and Powles, 2014b; Han et al., 2021). Considering the complexity of metabolic herbicide resistance and the diversity and number of P450s in plants, we hypothesize that the differences observed in metabolism speed and metabolite amounts between the R1 and the R2 populations could be explained by herbicide and environmental selection pressures, particularly the history of herbicide applications in the fields where R populations were collected (Yu and Powles, 2014b; Hada et al., 2020). Owing to the genetic diversity of the weedy species, differences in the P450 activity and presence of other genes may be involved in the resistance of *G. coronaria* to imazamox, which can be observed even in different individuals of a single population as reported by Yu and Powles (2014b). This ability of P450 to metabolize existing and yet-to-be-discovered herbicides is a serious threat to herbicide sustainability. It is unpredictable and could confer resistance to *G. coronaria* regardless of the herbicide molecule or MoA. This may include SAH, which are very effective in managing this newly resistant weed as reported recently (Hada et al., 2020). It has already been suggested that a common P450 can metabolize SAH and imazamox in multiple herbicide-resistant *P. rhoeas* (Torra et al., 2021).

Even no evidence was found on the presence of enhanced metabolism to tribenuron. Both R populations were able to metabolize, in part, imazamox, which belongs to the same MoA. It suggests that only one unit from the P450 system might contribute to the imazamox detoxification. This raises the hypothesis that enhanced metabolism is present in *G. coronaria* populations as a mechanism of resistance to herbicides. However, it could be hidden under the high resistance conferred by point mutations in the target gene. This observation is similar to the aforementioned reports on *P. rhoeas* (Rey-Caballero et al., 2017) and *R. rugosum (*Hatami et al., 2016), with multiple resistance mechanisms.

CONCLUSION

In this study, both point mutations in ALS gene and enhanced metabolism were confirmed as mechanisms endowing resistance to ALS-inhibiting herbicides in *G. coronaria*, a weed spreading in the most important cereal land in Tunisia. Amino acid substitutions could explain the high resistance levels for tribenuron herbicide and cross-resistance to imazamox, florasulam, and bispyribac. Meanwhile, enhanced metabolism was only detected for imazamox herbicide in both tested R populations, showing that *G. coronaria* weed is able to detoxify herbicides never received before. Therefore, the management of these weed R populations with NTSR mechanisms is challenged as reported by previous researches (De Prado and Franco, 2004; Yu et al., 2009; Yu and Powles, 2014a). So far, the relative importance of enhanced metabolism in the resistance response of *G. coronaria* to ALS inhibitors in field conditions is unknown. Furthermore, we ignore the interaction between TSR and enhanced metabolism, at both physiological and genetic levels. This should trigger more research, exploring the multiple resistance factors by means of transcriptome analyses and inheritance studies to understand the potential risk of the multiple resistance mechanisms evolving in *G. coronaria* in wheat fields.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AR-D performed metabolism experiment and ALS activity assay, while JM conducted absorption/translocation assay. ZH and JT performed dose–response experiment, participated in absorption/translocation experiment, and drafted the manuscript. CP-B elaborated the Translocation Phosphor image. YM performed ALS gene extraction and amplification, and worked on sequencing results. All authors reviewed and contributed to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.626702/full#supplementary-material

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