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Non-Target-Site Based Tolerance to Herbicides in Amaranthus palmeri

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Non-Target-Site Based Tolerance to Herbicides in *Amaranthus palmeri*

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by

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ABSTRACT

Palmer amaranth, one of the most aggressive and damaging broadleaf weeds in the USA, has evolved resistance to multiple herbicide modes of action. The overall objective of this research was to elucidate the mechanisms by which Palmer amaranth adapt to herbicide selection stress. This research aimed (1) to evaluate the efficacy of fomesafen, glufosinate, glyphosate and trifloxysulfuron to *Amaranthus* populations; (2) identify candidate genes for endowing tolerance to glufosinate; (3) investigate the involvement of non-target-site resistance (NTSR) mechanism in an ALS-resistant population; and (4) to examine the molecular basis of resistance to PPO inhibitors in Palmer amaranth populations from Arkansas. For objective 1, a total of 124 populations were collected in Arkansas between 2008 and 2015. Overall, 33%, 81%, and 100% of the populations were resistant to fomesafen, glyphosate, and trifloxysulfuron, respectively. Thirty percent of the populations were multiple resistant to fomesafen, glyphosate, and trifloxysulfuron. All populations were controlled ≥88% by glufosinate. For objective 2, the transcriptomes of glufosinate-tolerant and –sensitive biotypes were assembled using RNA-Seq. Thirteen candidate non-target genes were highly expressed in glufosinate-tolerant biotypes, including glutathione S-transferase (GST), two cytochrome P450, and nine additional genes related to stress signaling and detoxification. Validation of differential gene expression by quantitative real-time PCR revealed increased expression CYP72A219 and GST in glufosinate-treated tolerant biotypes, indicating their involvement in glufosinate tolerance. For objective 3, a population with cross resistance to multiple ALS-inhibiting herbicides was investigated. Two of the nine resistant plants harbored $Ser_{653}Asn$ mutation in the *ALS* gene. Resistant plants that lacked ALS mutations had elevated levels of CYP81B and GSTF10 genes. This Palmer amaranth population from Arkansas exhibit both target-site (TS) and NTSR to ALS inhibitors. For

objective 4, resistance to PPO inhibitors was first detected in a population collected in 2011 with resistance attributed to PPO Gly210 deletion. Several PPO-resistant populations were confirmed in 2014 and 2015; the majority (55%) of the resistant biotypes carried the same mutation. An alternative target-site mutation $Arg_{128}Gly$ was also identified in at least one population. Overall, this research showed that Palmer amaranth has multiple genetic adaptation traits to counteract the lethal effects of herbicides.

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CHAPTER I

INTRODUCTION

Introduction

Palmer amaranth (*Amaranthus palmeri* S Watson) is one of the most common, troublesome, and economically damaging agronomic weeds throughout the southern US (Ward et al., 2013). The competitive ability of Palmer amaranth is attributed to its fast growth rate (Jha et al., 2008), high fecundity (Keeley et al., 1987), good light interception, and high water use efficiency (Ehleringer, 1983). With estimates of over 600,000 seeds per plant, it can replenish the seedbank tremendously (Keeley et al., 1987). Because it competes effectively with crops for nutrients, water, light and space, it can significantly reduce crop yield. Palmer amaranth densities of 8 and 9 plants/m^2 can reduce soybean (*Glycine max*) yield by 78% (Bensch et al., 2003) and corn (*Zea mays*) grain yield by 91% (Massinga et al., 2001), respectively. Palmer amaranth interference for 63 d after crop emergence can cause 77% yield loss in cotton (*Gossypium hirsutum*) (Fast et al., 2009). Apart from reducing yield, heavy infestation of Palmer amaranth interfere with crop harvest and increased harvesting time by two- to four-fold (Smith et al., 2000).

Palmer amaranth control has become a challenge because of limited herbicide resources and its remarkable tendency to evolve herbicide resistance. As a dioecious species, Palmer amaranth is outcrossing, allowing herbicide resistance to spread rapidly (Steckel, 2007). Glyphosate resistance trait can be transferred up to 300 m through pollen from glyphosate-resistant males to glyphosate-susceptible female plants (Sosnoskie et al., 2012). To date, Palmer amaranth has been confirmed resistant to six different modes of action: acetolactate synthase (ALS) inhibitors, dinitroanilines, triazine, glyphosate, hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors, and most recently to protoporphyrinogen oxidase (PPO) inhibitors (Heap 2018). This narrows down available control option for resistant populations. PPO herbicides like

Valor and Reflex are intensively used to combat herbicide-resistant Palmer amaranth. Losing these herbicides to resistance problems would severely hamper efforts to manage weeds. Upcoming herbicide-resistant (HR) crop technology with resistance to glyphosate and dicamba, to glufosinate, to bleacher herbicides and other trait combinations are being developed. However, several weed species already reported resistance to some of these herbicides (Heap 2018). Knowing the response of Palmer amaranth populations to alternative herbicides in HR crops will refine herbicide recommendations for resistance management.

Herbicide resistance is the inherited ability of the plant to survive and reproduce following exposure to a dose of herbicide that would normally be lethal to the wild type (WSSA, 1998). Resistance is essentially a natural phenomenon which occurs spontaneously in weed populations, but is only noticed when a selection pressure is applied to the weeds through the application of a herbicide. Resistance to herbicides is the result of weed evolutionary adaptation to herbicide application (Delye, 2013). There are two primary mechanisms of herbicide resistance in weeds: target-site and non-target-site resistance. Target-site resistance (TSR) is caused by alteration of the gene encoding the target protein of the herbicide. Non-target-site resistance (NTSR) is due to mechanism(s) that minimize the amount of herbicide reaching the target site (Powles and Yu, 2010).This includes decreased herbicide penetration and translocation, increased herbicide sequestration, and metabolism. A study reported that rigid ryegrass resistance to nine modes of action is attributed to NTSR (Burnet et al., 1994). Previous research has indicated that low-dose herbicide application tend to promote non-target-site metabolic resistance (Gardner et al., 1998). Compared with target-site resistance, non-target-site herbicide resistance poses a greater threat because it can confer unpredictable resistance to herbicides with various modes of action and the multi-gene involvement in the mechanism (Petit

et al., 2010). Despite the importance of NTSR in understanding herbicide resistance evolution, the genetic determinants of NTSR are poorly understood because of the inherently complicated biochemical processes and the limited genomic information available in weedy species (Delye, 2013; Yuan et al., 2007). However, the recent advances in genomics technologies will enable identification of specific genes that mediate NTSR mechanism.

Acetolactate synthase inhibitors have been widely used for Palmer amaranth control since their introduction in 1982 (Gaeddert et al., 1997). Inhibition of ALS enzyme affects the synthesis of branched-chain amino acids (valine, leucine, and isoleucine), ultimately leading to plant death. There are five chemical families of herbicide that inhibit ALS: sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), sulfonylaminocarbonyltriazolinones (SCTs), pyrimidinylthiobenzoates (PTBs). The ALS herbicides are widely used because they provide broad-spectrum weed control at low doses, soil residual activity, wide application windows, excellent crop safety and low mammalian toxicity. However, these herbicides have high propensity to select resistant weed populations due to its widespread usage, strong selection pressure, and high levels of natural variability in the *ALS* gene (Tranel and Wright, 2002). To date, 160 species including Palmer amaranth are resistant to ALS-inhibiting herbicides (Heap 2018). Populations of Palmer amaranth resistant to ALS inhibitors are prevalent and have been identified in 14 states in the US, including Arkansas, Arizona, Delaware, Florida, Georgia, Illinois, Kansas, Maryland, Michigan, Mississippi, Tennessee, North Carolina, South Carolina, and Wisconsin (Nandula et al., 2012; Salas-Perez et al., 2017; Sosnoskie et al., 2011). Resistance is frequently attributed to target-site mutation. However, various studies have reported the presence of both target-site and non-target-site resistance in some populations of Palmer

amaranth, Spanish corn poppy (*Papaver rhoeas*) and flixweed (*Descurainia Sophia* L.) populations (Nakka et al., 2017; Rey-Caballero et al., 2017; Yang et al., 2016).

The widespread distribution of glyphosate-resistant weeds compelled farmers to use alternative herbicides including another non-selective herbicide, glufosinate, to control herbicide-resistant weeds in glufosinate-tolerant crops. Glufosinate is a fast-acting postemergence herbicide that controls weeds by inhibiting glutamine synthetase (GS) (E.C. 6.3.1.2), the enzyme that converts glutamate and ammonia to glutamine (Wild and Manderscheid, 1984). Inhibition of GS by glufosinate leads to ammonia accumulation, inhibition of amino acid synthesis, and indirect inhibition of photosynthesis, ultimately leading to plant death (Tachibana et al., 1986). To date, resistance to glufosinate has been confirmed in goosegrass (*Eleusine indica*) from Malaysia (Jalaludin et al., 2010; Seng et al., 2010) and Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Oregon (Avila-Garcia and Mallory-Smith, 2011). An amino acid mutation in the chloroplast-encoded GS gene, $Asp_{171}Asn$, conferred resistance to glufosinate in Italian ryegrass (Avila-Garcia et al., 2012). Resistance to glufosinate in glufosinate-resistant crops is achieved using transgenic methods to insert the *bar* or *pat* (phosphinothricin acetyltransferase) gene from a bacterium to the plant's genome, allowing detoxification of glufosinate by acetylation (Droge-Laser et al., 1994).

PPO-inhibiting herbicides became widely used during 1980-1990. However, after introduction of glyphosate-resistant crops, usage of PPO-herbicides declined until 2006 when economically damaging weeds evolved resistance to glyphosate (USDA-NASS). These current situations in agriculture reinforce the high interest in PPO-inhibiting herbicides in recent years (Dayan et al., 2010; Salas et al., 2016; Salas-Perez et al., 2017). The PPO enzyme catalyzes the oxidation of protoporphyrinogen IX (protogen) to protoporphyrin IX (proto), the last common

intermediate for the tetrapyrole synthesis system (Becerril and Duke, 1989; Jacobs et al., 1991). Several classes of PPO-inhibiting herbicides such as diphenyl ethers, thiadiazoles, oxadiazoles, triazolinones, N-phenyl-phthalimides, and pyrimidinediones inhibit the PPO enzyme. Inhibition of PPO results in the generation of singlet oxygen species that oxidize lipid and protein membranes, leading to plant death (Sherman et al., 1991). However, a few species of weeds have slowly evolved resistance from the repeated use of these herbicides in past few years. To date, 11 weed species have evolved resistance against PPO-herbicides including Palmer amaranth (Heap 2018; Salas et al., 2016; Salas-Perez et al., 2017;Giacomini et al., 2017). Many molecular and physiological mechanisms underlie the PPO-herbicide resistance, but most often, the evolution of resistance attributed to target-site mutations (Dayan et al., 2010; Edwards, 1996; Skipsey et al., 1997; Thinglum et al., 2011; Wuerffel et al., 2015).

Palmer amaranth control is becoming more difficult due to its adaptability, high seed production and resistance to many herbicides used for its management. With the evolution of Palmer amaranth populations that are resistant to herbicides of different modes of action, new approaches should be implemented to control and reduce the frequency of herbicide-resistant weeds. Understanding the molecular mechanisms endowing herbicide resistance will contribute to wiser use of herbicide resources and enable innovations that, together with integrated control strategies, will help minimize and manage herbicide-resistance evolution (Powles and Yu, 2010). An understanding of the fundamental molecular mechanisms behind herbicide resistance is necessary to minimize and manage resistance evolution and increase crop yield. With the recent advances in next-generation sequencing, tools are available for characterizing genome-wide gene expression in Palmer amaranth using transcriptomics, which is helpful in gene discovery and understanding the molecular mechanisms involved in plant response to herbicides and herbicide

resistance evolution. The goal of this study is to elucidate the mechanisms by which Palmer amaranth (or other weeds) adapt to herbicide selection stress. The specific objectives of this research are to (1) evaluate the efficacy of foliar-applied fomesafen, glufosinate, glyphosate and trifloxysulfuron to *Amaranthus* accessions collected between 2008 and 2015 in Arkansas; (2) identify candidate genes for endowing tolerance to glufosinate; (3) investigate the involvement of non-target-site resistance mechanism in an ALS-resistant population; and (4) to examine the molecular basis of resistance to PPO inhibitors in Palmer amaranth populations from Arkansas.

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CHAPTER II

REVIEW OF LITERATURE

Review of Literature

Palmer amaranth

Palmer amaranth (*Amaranthus palmeri* S Watson, subgenus *Acnida*, subsection *Saueranthus*) is one of the most common and problematic weed species in corn (*Zea mays*), cotton (*Gossypium hirsutum*), grain sorghum (*Sorghum bicolor*), peanut (*Arachis hypogea*), and soybean (*Glycine max*) in the US. It is described as a tall (usually about 2.5 m tall), erect, branched summer annual plant with leaves arranged alternately on the stems. The pistillate and staminate flowers occur in separate plants (dioecious species) in long, narrowly elongated terminal inflorescence**.** The female inflorescences have sharp bracts which are distinct from male inflorescences, which are soft to touch. Because it is a dioecious species, Palmer amaranth is an obligate outcrosser (Ward et al., 2013). Palmer amaranth can also hybridize, to a limited extent, with spiny amaranth, which increases the risk of spreading herbicide resistance to other *Amaranthus* species (Gaines et al., 2012).

Palmer amaranth is a C4 dicot with high rates of photosynthesis (81 μmol m^{-2} s^{-1}) compared to other C4 monocots such as corn and C3 dicots like cotton and soybean (Ehleringer, 1983; Gibson, 1998)**.** Under ideal conditions, Palmer amaranth can grow up to 5 cm daily. (Horak and Loughin, 2000). Temperature plays an important role in the net rate of photosynthesis in Palmer amaranth, and optimum photosynthesis occurs between 36-46°C (Ehleringer, 1983). Furthermore, Palmer amaranth exhibits diaheliotropism, meaning the plant is capable of tracking sunlight where the leaves orient themselves perpendicularly to the sun rays to maximize light interception and thus photosynthesis (Ehleringer and Forseth, 1980).

Infestation of Palmer amaranth significantly affects growth and yield of most agronomic crops, cotton being one of the most sensitive commodities. Cotton yield decreased by 5.38% for

every one additional Palmer amaranth plant per 9.1 m of row (Morgan et al., 2001). Palmer amaranth interference can cause significant yield reduction in soybean (Bensch et al., 2003), corn (Massinga et al., 2001), peanut (Burke et al., 2007), and sweet potato (*Ipomoea batatas*) (Meyers et al., 2010) crop productions. The success of Palmer amaranth as troublesome weed is attributed to its extended emergence period (Jha and Norsworthy, 2009), high fecundity (Keeley et al., 1987), rapid growth rate (Jha et al., 2008) with a C4 photosynthetic pathway (Ehleringer, 1983), genetic diversity, ability to tolerate adverse conditions, and its facility for evolving herbicide resistance (Ward et al., 2013).

Palmer amaranth can be controlled by timely applications of preemergence (PRE) and postemergence (POST) herbicides. Some of the PRE herbicides labeled on different crops for Palmer amaranth control are diuron (e.g. corn, cotton, grain sorghum), fluometuron (e.g. cotton, sugarcane), fomesafen (e.g. soybean, cotton), metribuzin (e.g. soybean), pendimethalin (e.g. corn, cotton, soybean, and many other crops), pyrithiobac and trifloxysulfuron(cotton), pyroxasulfone (e.g. corn, cotton, soybean, wheat), saflufenacil (e.g. corn, cotton, alfalfa), *S*-metolachlor (e.g. corn, cotton, soybean, and many other crops), and tembotrione (e.g. corn) (Scott et al. 2017). Labelled post-emergence herbicides for Palmer amaranth control include atrazine (e.g. corn, sorghum), dicamba (e.g. corn, sorghum), fomesafen (e.g. soybean), glufosinate (e.g. LibertyLink® crops), glyphosate (e.g. Roundup Ready® crops), and mesotrione (e.g. corn, sorghum) (Norsworthy et al., 2008).

The problem of Palmer amaranth escalated with the evolution of herbicide-resistant populations due to intensive use of herbicides. As a dioecious species, Palmer amaranth is outcrossing, allowing herbicide resistance to spread rapidly (Steckel, 2007). The glyphosate resistance trait was transferred across a 300-m distance through pollen from glyphosate-resistant

males to glyphosate-susceptible female plants (Sosnoskie et al., 2012). Resistance to six different modes of action (MOA) has been documented in Palmer amaranth: acetolactate synthase (ALS) inhibitors, dinitroanilines, triazine, glyphosate, hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors, and protoporphyrinogen oxidase (PPO) inhibitors (Heap 2018). Some Palmer amaranth populations are resistant to more than one MOA (Burgos et al., 2001). The rampant occurrence of ALS- and glyphosate-resistant Palmer amaranth populations has raised great challenge to farmers and researchers as this curtails the use of ALS inhibitors and glyphosate in crop production systems. With glyphosate and ALS inhibitors out of the weed management toolbox, weed control has returned to multiple-herbicide programs including PSII, PPO, and shoot/root inhibitors. Genetically modified crops with tolerance to 2,4-D and dicamba are recently commercialized and new technologies on HPPD-tolerant soybean (mesotrione and isoxaflutole) will be available soon. These alternative modes of action should be wisely used to preserve the utility of these herbicides and delay the evolution of resistant weeds.

Non-target-site-based resistance mechanism

There are two primary mechanisms of herbicide resistance in weeds: 1) target-site resistance (TSR) which is caused by alterations in the gene of the herbicide target site causing a decrease in the affinity of the herbicide for its target and 2) non-target-site resistance (NTSR) which is endowed by mechanisms not belonging to TSR (Delye, 2013). NTSR mechanisms include mechanisms that minimize the amount of herbicide in reaching the target site such as decreased herbicide uptake and translocation, increases herbicide sequestration, and metabolism (Powles and Yu, 2010). NTSR can confer unpredictable resistance to multiple herbicides with different MOA, including herbicides not yet used (Petit et al., 2010a; Petit et al., 2010b).

Sublethal dose of herbicide has been implicated to promote non-target-site metabolic resistance (Neve and Powles, 2005; Yuan et al., 2007). NTSR within a single plant is generally endowed by a combination of mechanisms controlled by different alleles as demonstrated in the segregation analysis of NTSR to ACCase ad ALS herbicides in progenies from controlled pairings of black-grass (*Alopecurus myosuroides*) parents (Petit et al., 2010b).

NTSR mechanisms as a part of plant stress response can interfere with herbicide penetration and accumulation at the target site, and/or protect the plant against the phytotoxic effects of herbicide action. Detoxification of herbicide usually follow a three-step process: 1) transformation of the herbicide molecule into a more hydrophilic metabolite by P450 monooxygenase, 2) conjugation of metabolites to glutathione or glucose, 3) further conjugation, cleaving and oxidizing and export of the molecule to the vacuole or cell wall for final degradation (Delye, 2013). Increased expression and activity of the enzymes that are involved in the detoxification process can contribute to reduced sensitivity of the plant to the herbicide. Reduced herbicide translocation may also be connected with detoxification of herbicides. Reduced translocation of glyphosate in horseweed (*Conyza candensis*) and ryegrass (*Lolium* spp) is due to rapid sequestration of glyphosate into the vacuole (Ge et al., 2012; Ge et al., 2010). Reduced herbicide absorption in an ACCase-resistant rigid ryegrass (*Lolium rigidum*) biotype is due to greater epicuticular wax density in the leaf cuticle (de Prado et al., 2005).

The basis for crop-weed selectivity for many herbicides is due to differential herbicide metabolism. The herbicide is safe for the crop because the crop metabolizes the herbicide whereas the weed does not. Enhanced herbicide metabolism is more common in grass weeds, but additional cases involving dicot weeds are emerging (Yu and Powles, 2014). The co-application of malathion, a P450 inhibitor, resulted in loss or decreased level of herbicide resistance in

several weed species demonstrated that enhanced herbicide metabolism is attributed to the cytochrome P450 family of proteins (Beckie et al., 2012; Breccia et al., 2017; Fu et al., 2017; Zhao et al., 2017). An enhanced herbicide metabolism has been reported in rigid ryegrass (Yu et al., 2013), wild mustard (*Sinapis arvensis*) (Veldhuis et al., 2000a), and late watergrass (*Echinochloa phyllopogon)* (Yasuor et al., 2009; Yun et al., 2005). Cytochrome P450s have been implicated in the metabolism of different herbicides in several crops and weed species (Cotterman and Saari, 1992; Hinz and Owen, 1996; Yu and Powles, 2014). The cloning of rice (*Oryza sativa*) CYP81A6 and Jerusalem artichoke (*Helianthus tuberosus*) CYP76B1 into *Arabidopsis* conferred resistance to sulfonylurea and phenylurea herbicides, respectively (Pan et al, 2006; Liu et al. 2012; Didierjean et al. 2002).

In addition to P450, glutathione S-transferases (GSTs) and glycosyltransferases have been implicated in herbicide metabolism (Yuan et al., 2007). Each of these three enzyme systems is encoded by large gene families. GSTs are ubiquitous in plants and have defined roles in herbicide detoxification (Wagner et al., 2002). Maize (*Zea mays*)and giant foxtail (*Setaria faberi*) are tolerant to atrazine due to its ability to rapidly detoxify the herbicide atrazine by glutathione conjugation with GST (Hatton et al., 1999; Hatton et al., 1996). Furthermore, induction of glutathione and GSTs by herbicide 'safeners' is employed commercially to protect crop plants from injury by certain herbicides (Farago et al., 1994). GSTs (phi and tau class) were identified to be involved in diclofop resistance in rigid ryegrass (Gaines et al., 2014). Furthermore, GSTA and GST1 were also recently reported to participate in non-target resistance or tolerance to ALS inhibitor in ryegrass and maize, respectively (Duhoux et al., 2015; Liu et al., 2015). A transgenic tobacco plant (*Nicotiana tabacum*) expressing maize GST1 had enhanced detoxification of alachlor (Karavangeli et al., 2005). In ACCase-resistant black-grass, GSTF1

catalyzed the conjugation of herbicide to glutathione and acted as peroxidases protecting the cell from oxidative damage (Cummins et al., 2009; Cummins et al., 1999; Cummins et al., 2013). Transgenic *Arabidopsis* expressing the black-grass *GSTF1*gene (*AmGSTF1*) has improved tolerance to some herbicides due to increased accumulation of protective flavonoids (Cummins et al., 2013).

Few non-target mechanisms have been elucidated at the molecular level because of the inherently complicated biochemical processes and limited genomic information available in weeds species (Yuan et al., 2007). Although NTSR mechanisms are widespread among weed species, its genetic determinant is poorly understood (Delye, 2013). The *de novo* transcriptome for waterhemp (*Amaranthus tuberculatus*) was characterized using GS-FLX 454 sequencing which can help in establishing valuable genomic resource for weed science research (Riggins et al., 2010). The transcriptome is useful for identifying potential candidate genes involved herbicide resistance and may serve as a reference for gene expression and functional genomics studies Using whole-transcriptome sequencing (RNA-Seq), several genes were identified in ryegrass, black-grass, flixweed (*Descurainia sophia* L.), and shortawn foxtail (*Alopecurus aequalis)* that confer NTSR to several herbicides (Gaines et al. 2014; Duhoux et al. 2017; Gardin et al. 2015; Yang et al. 2016; Zhao et al. 2017). Identifying NTSR genes will help in understanding NTSR evolution as well as help in understanding weed adaptation to herbicides.

Glufosinate

Glufosinate is a broad-spectrum, non-selective, post-emergence herbicide commonly used to control weeds in vineyards, orchards and genetically modified crops with LibertyLink® trait. Glufosinate inhibits glutamine synthetase (GS) (E. C. 6.3.1.2) enzyme which catalyzes the

conversion of glutamic acid and ammonia to form glutamine, the first reaction in the ammonia assimilation in higher plants. Glutamine synthetase functions as the major assimilatory enzyme for ammonia produced from nitrogen fixation, and nitrate or ammonia nutrition, as well as reassimilates ammonia released as a result of photorespiration and the breakdown of proteins and nitrogen transport compounds (Miflin and Habash, 2002). Inhibition of GS enzyme leads to rapid ammonia accumulation and amino acid biosynthesis inhibition which cause damage in the chloroplast structures and terminates photosynthetic activity, ultimately leading to plant death (Tachibana et al., 1986). The availability of glufosinate-tolerant cotton, soybean, and corn varieties allows farmers to use glufosinate in controlling broad spectrum of weeds without damaging the crop as well as provides growers an alternative herbicide for controlling Palmer amaranth that is resistant to other herbicides.

Despite the non-selective nature of glufosinate, variable control of goosegrass (*Eleusine indica*), large crabgrass (*Digitaria sanguinalis*) and Palmer amaranth has been observed (Beyers et al., 2002; Corbett et al., 2004; Everman et al., 2007). Differential tolerance to glufosinate in weed species has been attributed to application rate, plant species, application timing, humidity, growth stage, and variations in the level of absorption and translocation (Everman, 2007). Resistance to glufosinate has been reported in goosegrass (Seng et al., 2010) and Italian ryegrass (Avila-Garcia and Mallory-Smith, 2011). In glufosinate-resistant Italian ryegrass (*Lolium perenn*e L. ssp *multiflorum*) population from Oregon, resistance is attributed to mutation of the GS gene in which aspartic acid was substituted to asparagine at the amino acid position 171 (Avila-Garcia et al., 2012). The overproduction of GS enzyme conferred resistance to glufosinate in rice lines (Tsai et al., 2006). Glufosinate-tolerant crops are developed by expressing phosphinothricin N-acetyltransferase (PAT), which can detoxify L-phosphinothricin

(glufosinate) by acetylation of the amino group (Droge et al. 1992). Although resistance to glufosinate in Palmer amaranth has not been reported, tolerance to glufosinate has been observed in a population of Palmer amaranth in Arkansas (Botha, 2012).

ALS-inhibiting herbicides

Acetolactate synthase, also known as acetohydroxyacid synthase or AHAS (E.C. 4.1.3.18), is the first enzyme in the biosynthesis of the branched chain amino acids Ile, Leu, and Val. Inhibition of ALS leads to depletion of these amino acids disrupting protein synthesis, thereby causing plant death (Whitcomb, 1999). There are five chemical families of ALS herbicides, namely: sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine sulfonanilides (TP), pyrimidinylthiobenzoates (PTB), and sulfonylaminocarbonyltriazolinone. The ALS herbicides were widely used because they provide broad-spectrum weed control at low doses, soil residual activity, wide application windows, excellent crop safety and low mammalian toxicity. However, these herbicides have high propensity to select resistant weed populations due to its widespread usage, strong selection pressure, and high levels of natural variability in the *ALS* gene. Selection of ALS-resistant weed populations became evident in 1987, only five years after the introduction of the first SU, with the discovery of chlorsulfuron-resistant prickly lettuce (*Lactuca serriola* L.) and kochia (*Kochia scoparia* L. Shrad) (Mallory-Smith et al., 1990; Primiani et al., 1990). In the United States, the first case of resistance to ALS-inhibiting herbicides in Palmer amaranth was reported in 1993 in Kansas and cross-resistance to five ALS herbicides was documented in Arkansas in 1994 (Heap 2018). Resistance to ALS inhibitors in Palmer amaranth is widespread throughout the United States. As a result, ALS-inhibiting herbicides are no longer effective on ALS-resistant populations of Palmer amaranth.

Most cases of ALS resistance are due to mutation in the *ALS* gene resulting in an enzyme that is less sensitive to herbicide binding. To date, 29 amino acid substitutions in ALS conferring herbicide resistance were identified at Ala_{122} (5), Ala_{205} (2), Arg_{377} (1), Asp_{376} (1), Gly_{654} (2), Pro_{197} (11), Ser_{653} (3), and Trp_{574}(4) in weed species (Heap 2018). Recent investigation on 20 Palmer amaranth populations from Arkansas revealed TSR mechanism involving Trp_{574}Ser mutation with a few cases of double mutations involving Ala_{122}Thr, Pro_{197}Ala or Ser_{653}Asn (Singh, 2017). Amino acid substitution at Pro_{197} conferred resistance to SUs with low or no cross-resistance to IMIs (Tranel and Wright, 2002), whereas substitutions at Ala_{122} or Ser_{653} conferred resistance to IMI herbicides with low-level resistance to SUs (Bernasconi et al., 1996; Devine et al., 1991). Substitution at Trp_{574} confers resistance to IMIs, PTBs, SUs, and TPs (Tranel and Wright, 2002), whereas Asp_{376}Gluand Ala_{205}Phe substitution confers resistance to all five chemical families of ALS inhibitors (Brosnan et al., 2016; Whaley et al., 2007).

An important mechanism of naturally occurring (as opposed to evolved) resistance to ALS inhibitors is detoxification of the active herbicide in the plant. Inherent selectivity of a particular ALS inhibitor in a given crop is based on the crops' ability to metabolize the herbicide to nonphytotoxic compounds rapidly enough to prevent lethal herbicide levels from reaching the target enzyme ALS (Saari et al. 1994). Among the more common detoxification reactions involved in crop tolerance to sulfonylureas are hydroxylation, O-dealkylation, and deesterification (Saari et al. 1994). Maize is tolerant to nicosulfuron, a sulfonylurea herbicide, because nicosulfuron is rapidly metabolized to 5-hydroxypyrimidinyl nicosulfuron, a herbicidally inactive derivative which is then conjugated to glucose (Brown et al. 1991). Similarly, flumetsulam, a triazolopyrimidine that is selective in cereals, maize, and soybeans, also owes its selectivity to metabolic detoxification. Tolerant plants oxidize flumetsulam to one

or more hydroxylated metabolites, and soybean produces an open pyrimidine ring metabolite (Swisher et al., 1991). This tolerance mechanism in crops also appears to be the same mechanism responsible for poor control of some weeds by certain ALS herbicides (Saari et al. 1994).

Non-target-site resistance to ALS inhibitors have been reported in some weeds species. Reduced absorption and translocation rarely underlay resistance to ALS inhibitors (Cruz-Hipolito et al., 2013; Poston et al., 2001; Veldhuis et al., 2000b) and in only few cases have they been reported as partial-resistance mechanism (Riar et al., 2013; White et al., 2002) . An enhanced herbicide metabolism has been reported in rigid ryegrass (Yu et al., 2013), wild mustard (Veldhuis et al., 2000a), and late watergrass (Yasuor et al., 2009; Yun et al., 2005). A late watergrass biotype with multiple herbicide resistance to bispyribac-sodium, fenoxaprop-ethyl, and thiobencarb exhibited higher P450 hydroxylation activity toward these herbicides than the susceptible biotype, which suggests the involvement of cytochrome P450 enzymes as a mechanism for resistance (Yun et al. 2005). Furthermore, resistance to penoxsulam in late watergrass is conferred by an enhanced ability to detoxify the herbicide via malathion-sensitive monooxygenases (Yasour et al. 2009). The P450 inhibitor malathion reverses chlorsulfuron resistance in rigid ryegrass (Yu et al. 2009). Several cytochrome P450 have been identified in ALS-resistant weeds, such as CYP72A254 (also known as CYPA72A219) in late watergrass (bispyribac-sodium) (Iwakami et al., 2014), CYP81D in black-grass (mesosulfuron and iodosulfuron) (Gardin et al., 2015); CYP94A1, CYP94A2, CYP71A4 and CYP734A6 in shortawn foxtail (mesosulfuron) (Zhao et al. 2017). In addition, many CytP450s conferring resistance to various herbicides have been documented in several major crop species, such as CYP81A6 in rice (Pan et al., 2006); CYP76B1 in Jerusalem artichoke (Didierjean et al., 2002), and CYP81A9 in maize (*Zea mays*) (Liu et al. 2015). The wheat (*Triticum aestivum*)

CYP71C6v1 expressed in yeast (*Saccharomyces cerevisiae*) is able to metabolize several sulfonylurea herbicides such chlorsulfuron and triasulfuron through phenyl ring hydroxylation (Xiang et al., 2006).

PPO-inhibiting herbicides

Herbicides that inhibit protoporphyrinogen oxidase (PPO) include lactofen and fomesafen herbicides. Although they have been used for many years in the control of broadleaf weeds, their use began to slowly decline in the late 1990s due to the massive adoption of glyphosate-resistant varieties (Riggins et al., 2010). However, the advent and continued increase of glyphosate-resistant weeds cause growers to once again rely on PPO herbicides as an alternative approach to control weeds. Even though resistance to PPO herbicide has been slow to evolve, it may be expected to occur in weed species that are under strong and continuous selection pressure.

The PPO enzyme plays a major role in the biosynthesis of chlorophyll and heme by catalyzing the oxidation of protoporphyrinogen (protogen) to porphyrin IX (Proto IX). Upon treatment with PPO-herbicides, susceptible plants accumulate protogen IX which is then transported to the cytoplasm where it spontaneously form Proto IX. In presence of light, Proto IX generates singlet oxygen that causes degradation of cell membranes leading cells to break open and ultimately to cellular death (Becerril and Duke, 1989; Jacobs et al., 1991; Sherman et al., 1991). However, few species of weeds have slowly evolved resistance from the repeated use of these herbicides in past few years. To, date 11 different weed species have evolved resistance to PPO-herbicides including Palmer amaranth (Heap 2018; Giacomini et al., 2017; Salas et al., 2016; Salas-Perez et al., 2017).

The resistance mechanism to PPO herbicides in tall waterhemp is not due to difference in herbicide absorption and translocation, or metabolism (Shoup and Al-Khatib, 2005) but due to a unique codon deletion of glycine at position 210 in the mitochondrial isoform of the PPO2 gene (Patzoldt et al., 2006). The same mutation appears to be the only resistance mechanism to PPO herbicides in various tall waterhemp populations in the United States (Lee et al., 2008; Wuerffel et al., 2015). This glycine deletion alters the binding domain of the enzyme without negatively affecting substrate affinity (Dayan et al., 2010). However, a novel PPO mutation, $Arg_{98}Leu$ was detected in common ragweed (*Ambrosia artemisiifolia*), and recently two new substitutions identified at the same position in Palmer amaranth as $Arg_{128}Gly$ and $Arg_{128}Met$ (numbering changed due to the presence of 30 amino acid extension in Palmer amaranth) (Giacomini et al., 2014; Rousonelos et al., 2012). Computational modeling revealed that replacement of this arginine with hydrophobic glycine removed important hydrogen-bonding interactions with acifluorfen, fomesafen, and sulfentrazone resulting in reduced binding of these inhibitors (Hao et al., 2014).

Non-target resistance to PPO inhibitors in weed species has not yet been reported although natural tolerance to PPO inhibitors in crops is usually due to enhanced herbicide degradation. Diphenyl ether herbicides are detoxified in soybean by homoglutathione conjugation (Skipsey et al., 1997). Similarly, tolerance to the diphenyl ether fluorodifen in peas is due to rapid conjugation with glutathione (Edwards, 1996).

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CHAPTER III

RNA-SEQ TRANSCRIPTOME ANALYSIS OF *AMARANTHUS PALMERI* WITH DIFFERENTIAL TOLERANCE TO GLUFOSINATE HERBICIDE

Abstract

Amaranthus palmeri (Amaranthaceae) is a noxious weed in several agroecosystems and in some cases seriously threatens the sustainability of crop production in North America. Glyphosate-resistant *Amaranthus* species are widespread, prompting the use of alternatives to glyphosate such as glufosinate, in conjunction with glufosinate-resistant crop cultivars, to help control glyphosate-resistant weeds. An experiment was conducted to analyze the transcriptome of *A. palmeri* plants that survived exposure to 0.55 kg ha^{-1} glufosinate. Since there was no record of glufosinate use at the collection site, survival of plants within the population are likely due to genetic expression that pre-dates selection; in the formal parlance of weed science this is described as natural tolerance. Leaf tissues from glufosinate-treated and non-treated seedlings were harvested 24 h after treatment (HAT) for RNA-Seq analysis. Global gene expression was measured using Illumina DNA sequence reads from non-treated and treated surviving (presumably tolerant, T) and susceptible (S) plants. The same plants were used to determine the mechanisms conferring differential tolerance to glufosinate. The S plants accumulated twice as much ammonia as did the T plants, 24 HAT. The relative copy number of the glufosinate target gene *GS2* did not differ between T and S plants, with 1 to 3 *GS2* copies in both biotypes. A reference cDNA transcriptome consisting of 72,780 contigs was assembled, with 65,282 sequences putatively annotated. Sequences of *GS2* from the transcriptome assembly did not have polymorphisms unique to the tolerant plants. Five hundred sixty-seven genes were differentially expressed between treated T and S plants. Of the upregulated genes in treated T plants, 210 were more highly induced than were the upregulated genes in the treated S plants. Glufosinate-tolerant plants had greater induction of ABC transporter, glutathione S-transferase (*GST*), NAC transcription factor, nitronate monooxygenase (*NMO*), chitin elicitor receptor

kinase (*CERK1*), heat shock protein 83, ethylene transcription factor, heat stress transcription factor, NADH-ubiquinone oxidoreductase, ABA 8'-hydroxylase, and cytochrome P450 genes (*CYP72A, CYP94A1*). Seven candidate genes were selected for validation using quantitative real time-PCR. While *GST* was upregulated in treated tolerant plants in at least one population, *CYP72A219* was consistently highly expressed in all treated tolerant biotypes. These genes are candidates for contributing tolerance to glufosinate. Taken together, differential induction of stress-protection genes in a population can enable some individuals to survive herbicide application. Elevated expression of detoxification-related genes can get fixed in a population with sustained selection pressure, leading to evolution of resistance. Alternatively, sustained selection pressure could select for mutation(s) in the *GS2* gene with the same consequence.

Introduction

Amaranthus palmeri is a dioecious, weedy *Amaranthus* species native to Southwestern North America [1, 2]. It is one of the most widespread, troublesome, and economically damaging weeds in agronomic crops throughout the southern United States [2]. Infestation of Palmer amaranth can cause from 70% to more than 90% yield loss in cotton (*Gossypium hirsutum*) [3], soybean (*Glycine max*) [4], and corn (*Zea mays*) [5]. *A. palmeri* is difficult to control because of its rapid growth rate, high fecundity, tiny seeds dispersed by multiple agents, continuous emergence pattern, high genetic diversity, high propensity for evolving herbicide resistance, and dioecious nature with long-distance pollen dispersal [1, 6-9]. To date, resistances to six herbicide mechanisms of action (MOAs) have been confirmed in *A. palmeri*: acetolactate synthase (ALS) inhibitors, 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors, enolpyruvyl shikimate-3-phosphate synthase (EPSPS) inhibitor (glyphosate), mitosis inhibitors (dinitroanilines), photosystem II inhibitors (triazines), and protoporphyrinogen oxidase (PPO) inhibitors [10]. The increasing resistance of *A. palmeri* to herbicides is a threat to corn, cotton, peanut (*Arachis hypogea*), and soybean production [1, 11-14]. Alternative management strategies are needed to combat this problem in several areas in North America [11, 15-18].

Herbicides are used as a major tool for controlling weeds and the evolution of herbicide-resistant (HR) weeds is an increasing issue worldwide. Glyphosate-resistant (GR) crops, first commercialized in 1996, were adopted quickly by growers because the technology allowed the use of the nonselective herbicide, glyphosate, in-season. The technology drastically simplified weed control with the use of a single, inexpensive, highly effective herbicide. In fact, GR crops constituted 80% of the 175 million ha planted with transgenic crops globally [19]. However, the over-reliance on glyphosate and its application over a vast land area has exerted unprecedented

selection pressure on weeds, resulting in the evolution of GR weeds including *A. palmeri*. Glyphosate-resistant *A. palmeri* was first identified in Georgia in 2004 [20] and subsequently became widespread across the South, Midwest and Northeast regions of the United States [10]. The widespread distribution of glyphosate-resistant weeds compelled farmers to use alternative herbicides including another non-selective herbicide, glufosinate, to control HR weeds in glufosinate-tolerant crops. Glufosinate is a fast-acting postemergence herbicide that controls weeds by inhibiting glutamine synthetase (GS) (E.C. 6.3.1.2), the enzyme that converts glutamate and ammonia to glutamine [21]. Inhibition of GS by glufosinate leads to ammonia accumulation, inhibition of amino acid synthesis, and indirect inhibition of photosynthesis, ultimately leading to plant death [22]. To date, resistance to glufosinate has been confirmed in *Eleusine indica* from Malaysia [23, 24] and *Lolium perenne* ssp. *multiflorum* from Oregon [25]. An amino acid mutation in the chloroplast-encoded GS gene, $Asp_{171}Asn$, conferred resistance to glufosinate in *L. perenne* ssp. *multiflorum* [26]. Resistance to glufosinate in glufosinate-resistant crops is achieved using transgenic methods to insert the *bar* or *pat* (phosphinothricin acetyltransferase) gene from a bacterium to the plant's genome, allowing detoxification of glufosinate by acetylation [27].

Differential responses to glufosinate in weeds have been attributed to several factors including light, temperature, humidity, growth stage, application rate, application timing, species, and variation in the level of herbicide absorption, translocation, and metabolism [28, 29]. Control of *A. palmeri* by glufosinate is variable [30-32]. A previous study reported higher uptake, mobility, and metabolism of glufosinate in *A. palmeri* compared to the more susceptible *Ipomoea lacunosa* [28]. As is commonly observed, differential tolerance to herbicides are often due to non-target-site (NTS) mechanisms, involving the detoxification of herbicide by

biochemical modification and/or the compartmentation of the herbicide and its metabolites [33]. Cases of weed resistance to herbicides due to NTS mechanisms have been increasing (www.weedscience.org). The genetic bases of NTS mechanisms are not fully understood due to the complex interaction of biochemical processes and limited genomic information on weedy species [33-35]. In this study, we investigated *A. palmeri* accessions with differential tolerance to glufosinate.

The genome of *A. palmeri* is not yet deciphered although the genome and transcriptome of its cultivated relative grain amaranth (*Amaranthus hypochondriacus*) was completed in 2014 [36]. Also recently, the transcriptome of two weedy species *Lolium rigidum* [37] and *Echinochloa crus-galli* [38] were sequenced to identify genes involved in herbicide resistance. Understanding the molecular mechanisms underlying herbicide resistance could be used to mitigate and manage resistance evolution and reduce weed impact on crops. This study assembled the transcriptome sequence of *A. palmeri* exposed to glufosinate compared to controls to elucidate candidate genes involved in differential tolerance to glufosinate.

Materials and Methods

Plant Materials

Amaranthus palmeri seed samples from 120 fields were collected in Arkansas, USA between 2008 and 2014. The collection of plant samples from the field was done with permission of farm owners, managers, consultants, or Extension Agents. In the process of collecting samples, no endangered species were affected. Inflorescences of at least 10 female plants per field were harvested, dried, threshed, and cleaned for herbicide bioassays in the greenhouse. One accession

of interest (08-Lee-C) was collected from a field that was planted with glyphosate-tolerant (Roundup Ready®) soybean in 2008 and glyphosate-tolerant cotton in 2006 and 2007. Although this field had no record of being sprayed with glufosinate, some plants survived exposure to glufosinate (0.55 kg ha^{-1}) in the greenhouse. The survivors were grown and allowed to cross-pollinate to produce the first cycle of purified (intercrossed) progeny (C1).

To study the potential survival mechanisms, seeds of 08-Lee-C and the C1 progeny were planted in 4-cm-diameter pots using commercial potting soil mix (Sunshine Mix, Bellevue, WA, USA). Seedlings (100) were grown at one plant per pot in a growth chamber maintained at 32/26 °C day/night temperature with a 16-h photoperiod. Plants were watered daily and fertilized with a water-soluble all-purpose plant food containing 15-30-15% NPK (Miracle-Gro®, Marysville, OH, USA), every 2 wk. Fifty plants per accession (9-cm tall) were sprayed with glufosinate (0.55 kg ai ha^{-1}) (Liberty®, Bayer) mixed with 20 g L^{-1} ammonium sulfate to identify S and T plants. Susceptible and T plants from the non-treated control were identified by ammonia accumulation assay. Six confirmed S plants from 08-Lee-C and T plants from C1 were used for ammonia accumulation assay, determination of chloroplast-encoded glutamine synthetase (*GS2*) copy number, and RNA-Seq experiment.

Phenotypic evaluation of *A. palmeri* response to glufosinate

The response of *A. palmeri*, collected between 2008 and 2014, was evaluated in the greenhouse. A known herbicide-susceptible accession (SS) was included in each experiment as control [39]. Five-hundred mg of seeds from each field-collected plant were mixed to make a composite seed sample representing each accession. The experiment was conducted twice in a randomized complete block design with two replications. Each replication consisted of one cellular tray (28 X 54 cm) with 50 cells (Redway Feed Garden and Pet Supply, Reedway, CA,

USA) filled with a commercial medium (Sunshine Mix, Bellevue, WA). Composite seeds from each accession were planted in each cell and seedlings were thinned to one per cell. Glufosinate was applied at 0.55 kg ha^{-1} when seedlings were 7.5- 9 cm tall using a laboratory sprayer fitted with a flat fan nozzle tip (800067 TeeJet, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha^{-1} at 269 kPa. The herbicide was applied with 20 g L^{-1} ammonium sulfate. The plants were assessed visually relative to the non-treated control 21 d after treatment (21 DAT) using a scale of 0 to 100, where 0 = no visible injury and 100% = complete desiccation. The number of survivors was recorded. Survivors from glufosinate treatment were grown to produce seed. Data were analyzed using hierarchal clustering in JMP Pro v. 12.

Herbicide resistance profiling of a selected *A. palmeri* accession

Data from the differential tolerance evaluation were used to select an accession for further study. Accession 08-Lee-C had the most number of survivors with minimum injury. Seeds from 08-Lee-C and SS accessions were planted as described in the previous section. Seedlings (7.5-9 cm tall) were treated with the recommended dose of fomesafen (264 g ha^{-1}) (Flexstar®, Syngenta), glyphosate (870 g ha^{-1}) (Roundup PowerMAX®, Monsanto), dicamba (280 g ha^{-1}) (Clarity®, BASF), and ALS inhibitors pyrithiobac (73 g ha^{-1}) (Staple LX®, DuPont) and trifloxysulfuron (8 g ha^{-1}) (Envoke®, Syngenta). The ALS inhibitors were applied with 0.25% non-ionic surfactant (Induce®, Helena Chemical Co., Collierville, TN, USA), respectively. Herbicide treatments were applied as described in the previous section. Mortality was evaluated 21 d after treatment. The experiment was conducted in a randomized complete block design as in the previous section. Data were analyzed using ANOVA in JMP Pro v. 12.

Evaluation of tolerance level to Glufosinate

A dose-response bioassay was conducted in the greenhouse to determine the tolerance level of 08-Lee-C and C1 relative to the SS accession to glufosinate. Seeds were sown in 15-cm diameter pots filled with commercial potting soil. Seedlings, 7.5-cm tall, were sprayed with 11 doses of glufosinate using a laboratory sprayer as described in the previous section. The 08-Lee-C and C1 accessions were sprayed with glufosinate from 0.0012 to 0.5940 kg ai ha^{-1}, whereas the SS accession was sprayed at 0.0006 to 0.5950 kg ha^{-1}, with a non-treated check. The herbicide was applied with 20 g L^{-1} ammonium sulfate. Shoot biomass was harvested 21 DAT, dried at 60°C for 72 h, and weighed. The experiment was conducted in a randomized complete block design with four replications. Five plants were used per replication (20 plants total) per herbicide concentration.

Data were analyzed using SAS JMP Pro v. 13 in conjunction with SigmaPlot v.13 (Systat Software, Inc., San Jose, CA, USA) for nonlinear regression analysis. The percentage biomass reduction was fitted to a nonlinear, sigmoid, four-parameter logistic regression model defined by

$$y = c + [(d - c)/(1 + e\{-a(x - b)\})]$$

where *y* represents the biomass reduction expressed as percentage relative to the non-treated control, *a* is the growth rate, *b* is the inflection point, *c* is the lower asymptote, *d* is the upper asymptote, and *x* is the glufosinate dose. The herbicide doses that would cause 50% growth reduction (GR_{50}) were estimated using the fitted regression equation.

Ammonia accumulation assay

To identify S and T plants without glufosinate treatment, a leaf disc assay was conducted to measure ammonia accumulation caused by the inhibition of photorespiration by glufosinate [40].

The assay was conducted using 50 non-treated plants each from 08-Lee-C and the C1 progeny. In addition, leaf tissues from three 08-Lee-C plants that were controlled (S) and three C1 plants that survived (T) glufosinate application at the whole plant level were also tested. From each plant, two leaf discs (5-mm diameter) were excised from the youngest, fully expanded leaf of 6.4-cm tall seedlings. One leaf disc was placed per well in a microtiter plate containing 200 µM glufosinate. The plate was sealed with micropore tape and placed on a bench under light for 24 h. The plate was moved to a -80 °C freezer to stop the reaction. After two freeze-thaw cycles, ammonia content was measured in a spectrophotometer (Pharma Spec UV-1700, Shimadzu, Columbia, MD) at 630 nm using a modified method by Molin and Khan [41]. Leaf discs from S plants were expected to have higher ammonia content than those from T plants.

Glutamine synthetase (*GS2*) relative copy number

Leaf tissues were harvested from confirmed S and T plants (three each) without glufosinate treatment and stored at -80°C until processing. Leaf tissues were harvested also from plants treated with glufosinate, 24 HAT. Upon evaluation of plant response 21 DAT, leaf tissues from three S and T plants were used also to determine the relative copy number of *GS2* gene. Genomic DNA was extracted using the modified hexadecyltrimethylammonium bromide (CTAB) method [42]. Quantitative real-time PCR (qPCR) was used to determine the genomic copy number of *GS2* relative to a housekeeping RNA dead box helicase gene *GS2* in *A. palmeri*. The primer pair GS2-F (5'- ATACGGAGAAGGAAGGCAAAG -3') and GS2-R (5'-TGTGGGTTCCCAAAGTAGTG-3') were designed to amplify a region of the chloroplast *GS.* RNA dead box helicase gene primers A36-F (5'- TTGGAACTGTCAGAGCAACC-3') and A36-R (5'-GAACCCACTTCCACCAAAAC-3') were used as internal primers to normalize the samples for any differences in DNA quantities. Reactions were conducted in three technical

replicates, and a negative control consisting of primer pairs with no template was included. An 8-fold serial dilution of genomic DNA samples, ranging from 0.00064 to 50 ng, was used to construct a standard curve. The slope of the standard curve was used to determine amplification efficiency (*E*). The qPCR reaction efficiency was 97% with an R^2 of 0.9907 and a slope of 3.271 indicating good assay validation. Genomic DNA templates (2 ng) were amplified in a 25-µL reaction containing 12.5-µL Bio-Rad iQ SYBR Green Supermix, 2-µL of primers (1:1 mix of forward and reverse primers), and nuclease-free water. Reaction conditions included 10 min incubation at 94°C, then 40 cycles of 94°C for 15 s and 60°C for 1 min, followed by a melt-curve analysis to confirm single PCR product amplification. Data were analyzed using CFX Manager software (v.1.5). Relative *GS2* copy number was calculated as $\Delta Ct = (Ct, A36\ - Ct, GS2)$ according to the method described by Gaines *et al* [43]. Increase in *GS2* copy number was expressed as $2^{\Delta Ct}$. Results were expressed as the fold increase in *GS2* copy number relative to *RNA dead box helicase*.

RNA-Seq Analysis

Sample preparation for RNA-Seq

This experiment used leaf tissues from non-treated and treated, confirmed S and T plants. These were the same plants used for ammonia assay and *GS2* copy number determination. Tissues were collected 24 h after glufosinate application for RNA extraction. This collection time was selected to capture herbicide stress adaptation genes and because maximum absorption of glufosinate occurs 24 HAT [28]. Treatments were designated as non-treated S (susceptible without treatment, SWO), non-treated tolerant (tolerant without treatment, TWO), treated susceptible (SWT), and treated tolerant (TWT) plants with three biological replicates. Total RNA

was extracted from young leaf tissues of S and T plants using PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. The extracted RNA was treated with DNase (Invitrogen, Carlsbad, CA, USA) to remove potential genomic DNA contamination, according to the manufacturer's instructions. The samples were then sent to the Clemson University Genomics and Computational Biology Laboratory, South Carolina for sequencing the transcriptome.

Transcriptome sequencing and assembly

Total RNA was normalized and converted to cDNA using the TruSeq RNA library kit v2.0 (Illumina). Final sequencing products were validated for size on an Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) and sequenced using a 2x125bp paired-end sequencing module on an Illumina HiSeq 2500 (Illumina). Raw sequence reads were assessed for quality using the FastQC software package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and preprocessed to remove sequence adapters and low quality bases with the Trimmomatic software [44]. A reference unigene assembly comprehensive of developmental stage, tissue source, and experimental conditions was prepared by concatenating all preprocessed reads and assembling with the Trinity software package [45]. The resulting unigene assembly was filtered for genuine coding sequences (e.g., sequences without internal stop codons or chimeras) with the TransDecoder software, and clustered by identity with the CD-HIT software [46] in an attempt to collapse homologs, but not paralogs, at high identity thresholds.

Differential gene expression (DGE)

Paired-end reads from each individual were aligned to the *de novo* transcriptome using the Subread package [47, 48]. Samtools was used to convert alignments from sam to bam format,

sort, and index [49, 50]. Subread's featureCounts counted the number of reads that aligned to each gene in the transcriptome [47, 48]. The final gene counts were loaded into Bioconductor's edgeR package for statistical analysis [51-55]. Variance between samples was visualized by a multidimensional scaling (MDS) plot. Volcano plots were generated for each comparison of samples. The criteria for differential gene expression included a fold-change ≥ 2 between compared groups and statistical significance at $P \leq 0.05$ [56]. Expression differences were compared between non-treated T and non-treated S (TWO vs SWO), treated T and treated S (TWT vs SWT), treated T and non-treated T (TWT vs TWO), and between treated S and non-treated S (SWT vs SWO).

Transcriptome annotation

The final reference assembly was annotated by blastx and blastp alignment to the non-redundant protein database at NCBI, the UniProt-swissprot database, and the uni-ref database to determine homology to known genes. Protein domains were determined by alignment to the HMMER database (http://hmmer.janelia.org). Signal peptides were determined with the SignalP software [57] and transmembrane regions predicted with tmHMM (http://www.cbs.dtu.dk/index.shtml). Gene ontology terms were derived from the best BLAST match [58] and clustered to determine enrichment using the Agbase tool [59].

The entire dataset was submitted as NCBI BioProject (PRJNA390774), which is a part of the U.S. National Library of Medicine of the National Institutes of Health. The 12 samples that were used to construct the transcriptome, and to run the differential gene expression comparisons were submitted as 12 separate BioSamples, SAMN07260017-SAMN07260028. The trimmed, paired-end fastq files for each of the 12 samples were submitted to the Sequence Read Archive, SRR5759376 – SRR5759387. Finally, the transcriptome was submitted to the Transcriptome

Shotgun Assembly Sequence Database. The transcriptome, consisting of 72,780 transcripts, is under TSA submission: SUB2788796.

Sequence analysis of the glutamine synthetase 2 (*GS2*) gene

Glutamine synthetase 2 (*GS2*) gene sequences of the T and S biotypes were extracted and assembled from the transcriptome data. A 1296-bp *GS2* gene (431 amino acids) from S and T plants was sequenced. The full length *GS2* sequences of S and T plants were aligned using Sequencher 5.4.6 and BioEdit software packages to identify amino acid substitutions. Sequence alignment also included *GS2* sequences of other *Amaranthus* species (*A. viridis, A. albus, A. spinosus, A. hybridus, A. lividus* and *A. thunbergii*) available in the database at http://www.weedscience.org.

Heat Map Analysis

Differentially expressed genes associated with abiotic stress response were subsampled and subjected to heat map analysis. Normalized read count averages were calculated to produce biological expression profiles followed by hierarchical clustering to recursively merge expression based on pair-wise distances between non-treated T (TWO), non-treated S (SWO), glufosinate-treated T (TWT), and glufosinate-treated S (SWT) samples. Digital expressions were visualized between rows for normalized read count numbers (minimum & maximum) expression patterns. The expression pattern was generated using GENE-E tool (http://www.broadinstitute.org/cancer/software/GENE-E/).

Selection of candidate non-target genes

Genes that were commonly expressed between treated S and T, and between treated T and non-treated T plants were selected for further evaluation based on their gene ontologies (GO).

Genes that were assigned with GO molecular function and biological process related to metabolism and signaling pathways (oxidoreductase activity, nuclear acid binding transcription factor activity, hydrolase activity, transferase activity, transmembrane transporter activity, transferase activity, protein transporter activity, biosynthetic process, small molecule metabolic process, signal transduction, homeostatic process, immune system process, cell wall organization, secondary metabolic process, nitrogen cycle metabolic process) were evaluated based on UniProt and their fold change. Contig assemblies that were consistently upregulated in the treated T (relative to treated S and non-treated T) with a significant P-value in the DESeq analysis were selected, for a total of 49 contigs. A subset of this list was generated based on known gene function. Contigs with predicted annotations related to stress response, signaling, transcription factors, and herbicide metabolism were selected as potential candidate NTS genes involved in glufosinate tolerance.

Candidate gene validation by qRT-PCR

Two *A. palmeri* populations were treated with glufosinate at 0.37 kg ha^{-1} using the previously described procedure. Leaf tissues were collected three days before and 24 h after herbicide treatment. Tolerant and susceptible plants from each population were identified three weeks after herbicide treatment based on level of injury. Three biological replicates from the non-treated and treated samples from each biotype within each population were used for the validation experiment. Total RNA was extracted from leaf tissues using PureLink RNA Mini kit (Life Technologies, Carlsbad, CA). Genomic DNA was removed using DNAse I (Thermo Scientific, Waltham, MA). cDNA was generated from 5 µg total RNA using Reverse Transcription System first-strand cDNA synthesis kit (Promega). A 4-fold serial dilution of cDNA samples (1:1, 1:5, 1:25, 1:125) was used to construct a standard curve. Seven of the candidate NTS genes were

subjected to real-time quantitative PCR with primers designed using Primer3 tool. Two genes (β-tubulin, RNA helicase) were used as internal controls for normalization of gene expression. Primers had an amplification efficiency of 96 to 110%.

The expression level of 7 candidate NTS genes was measured in 24 plants. Quantitative real-time PCR reactions were conducted in a 12-µL volume containing 6.25 µL of SyberGreen Master Mix, 1 µL of 1:25 diluted cDNA, and 0.5 µL of 10 µM primers (1:1 mix of forward and reverse primers). Amplification was performed in a Bio-Rad MiniOpticon System PCR machine (CFX96, Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: 10 min at 94 ºC, 40 cycles of 94 ºC for 15 s and 60 ºC for 1 min, followed by a melt-curve analysis to confirm single PCR product amplification. Data were analyzed using CFX Manager software (v.1.5). Slopes for target and internal control genes were equivalent as observed in amplification plots. Comparative CT method was used to calculate relative expression levels as $2^{-\Delta\Delta Ct}$ where ΔCT = [CT target gene–geometric mean (CT internal control genes)] and ΔΔCT = [ΔCt tolerant - ΔCt susceptible]. Wilcoxon non-parametric test (α=0.05) was used to determine statistical difference in gene expression between tolerant and susceptible biotypes.

Results

Differential response of *A. palmeri* accessions to glufosinate

The majority of accessions were 100% sensitive to 0.55 kg ha^{-1} glufosinate, except for some, which had few survivors. The 120 accessions differentiated into three groups based on mortality and levels of injury of survivors (Table 1). The first group is composed of the 88 most sensitive accessions. The second group, composed of 28 accessions, were controlled 94 to 99% with survivors incurring 60-99% injury. The third group consisted of four apparently segregating

accessions with 88 to 97% mortality. Survivors from these accessions incurred 30-80% injury. Of the possibly segregating accessions, only survivors from 08-Lee-C were able to produce sufficient seeds to continue further experiments and evaluation. Seven percent of 08-Lee-C survived glufosinate treatment, of which 4% of treated plants had <61% injury and produced seeds. Survivors from other recalcitrant accessions were not able to produce enough seeds due to either having high injury (>75%) or low number of survivors, which were all males. Considering that plants growing in the field tend to be more robust than those in the greenhouse, the likelihood of expected escapes in the field may be higher than that in the greenhouse. Plants in the field also do not receive uniform amounts of herbicide for various reasons such as partial coverage by other plants or differential plant size. In addition, plants maintained in the greenhouse that are well-watered and cultured under warm temperatures grow faster and reach the recommended spraying heights earlier than those growing in the field due to less variation in ambient conditions in the greenhouse [60].

Response of 08-Lee-C to other foliar herbicides

The 08-Lee-C accession was susceptible to dicamba and fomesafen, but resistant to glyphosate and ALS inhibitors, which are commonly used herbicides. The mortality of 08-Lee-C was 98% and 99% with fomesafen and dicamba, respectively (Table 2). On the other hand, 08-Lee-C was controlled poorly with glyphosate (EPSPS inhibitor) (61%) as well as with ALS inhibitors trifloxysulfuron (25%), and pyrithiobac (21%). This accession is resistant to two commonly used modes of action, thus limiting the herbicide options for post-emergence weed mitigation.

Resistance level to glufosinate

The response of *A. palmeri* to 11 doses of glufosinate fitted a sigmoidal, logistic function (Fig. 1). The glufosinate doses required to reduce growth by 50% (GR_{50}) were 0.076, 0.110, and 0.214 kg ha^{-1} for SS, 08-Lee-C, and C1 accessions, respectively (Table 3). Based on these GR_{50} values, the level of tolerance to glufosinate in 08-Lee-C and and C1 accessions was 1.4- and 2.8-fold, respectively, relative to the susceptible standard (SS). The GR_{50} increased 2-fold, from 110 g ha^{-1} in 08-Lee-C to 214 g glufosinate ha^{-1} in C1.

Ammonia accumulation in response to glufosinate

Glutamine synthetase, the target site of glufosinate, is a nuclear-coded enzyme that catalyzes the conversion of L-glutamate to L-glutamine by assimilating ammonia in the cytoplasm and plastids, but predominantly in the chloroplast of green tissues [21]. Ammonia accumulation is a direct response to the inhibition of this pathway by glufosinate. The ammonia concentration in S plants was 830 µg g^{-1} fresh leaf tissue ($\pm$60) and was 394 µg g^{-1} fresh weight ($\pm$40) in T plants (Fig. 2). The S plants accumulated 2X more ammonia than the T plants, indicating rapid depletion of functional glutamine synthetase as a consequence of glufosinate treatment.

Glutamine synthetase 2 (*GS2*) relative copy number

The relative *GS2* copy number of S and T plants ranged from 1 to 3 (Fig. 3). Similar *GS2* copies were detected in both S and T plants, indicating that differential tolerance to glufosinate is not due to amplification of the *GS2* gene. Transcriptome analysis also revealed that *GS2* was not

differentially expressed between T and S plants, which indicated that differential tolerance to glufosinate in *A. palmeri* was not due to changes in expression of the target enzyme.

Glutamine synthetase gene sequence analysis

Glutamine synthetase plays a primary role in plant nitrogen metabolism by catalyzing the conversion of glutamate to glutamine [21]. Glutamine synthetase in higher plants exists in two major isoforms: GS1 in the cytosol and GS2 in the chloroplast/plastids [61]. The cytosolic form (GS1) is the predominant isoform in roots and non-green tissues [62]. The chloroplast form of glutamine synthetase (GS2) is the major isoform in leaves, which is primarily responsible for recycling ammonia during photorespiration and synthesis of glutamine [63]. In our study, two different alleles of *GS2* were observed in the T plants and one in the S plants. The nucleotide sequences obtained from the two biotypes had 97-99% identity with *GS2* sequences from other *Amaranthus* species (*A. albus, A. hybridus, A. spinosus, A. lividus, A. thunbergii, and A. viridis*). The T biotype differed in six amino acids in the upstream region of *GS2* when compared to the S biotype. Seven nonsynonymous point mutations ($Tyr_{8}Asn$, $Ser_{25}Leu$, $Asn_{26}Ser$, $Lys_{37}Gln$, $Gly_{39}Lys$, $Gln_{54}Lys$, $Asp_{56}Glu$) in the upstream region were detected in one of the *GS2* alleles of the T biotype (Fig. 4). The second allele of the T biotype harbored only the $Tyr_{8}Asn$ substitution. These nonsynonymous substitutions identified in the *A. palmeri* T biotype also occur in herbicide-susceptible *A. viridis*, indicating that these substitutions do not contribute to tolerance to glufosinate. Other nucleotide polymorphisms between T and S plants were synonymous mutations.

Global transcriptional changes in *A. palmeri* 24 h after glufosinate application

A reference cDNA transcriptome consisting of 72,794 sequences was assembled (Table 4). Treatment samples were similar as indicated in the multidimensional scaling (MDS) plot (Fig. 5). Biological replicates from the same treatment clustered together indicating low bias and variation among treatment samples. One or more GO terms were assigned to 33,516 sequences with 76,455 GO assignments in total for biological process (31.9%), cellular component (10.8%) and molecular function (57.3%) categories.

Background differences between susceptible and tolerant plants. Pairwise comparison between non-treated S and T plants showed 438 differentially expressed genes, 158 of which were downregulated and 280 were upregulated in the T plants relative to S plants (Table 5). Genes that were notably more expressed in the T plants relative to the S plants without herbicide treatment included cytochrome P450s (*Cyp72A219, Cyp86b, Cyt77A, Cyt71A, Cyt76A, Cyt86A*), transporters (ABC transporter), transferases (glycosyltransferase, acyltransferases), antioxidants (glutathione-S-transferase, superoxide dismutase), and genes related to lipid metabolism (esterase lipase).

Genes induced by glufosinate application. Relative to the respective non-treated checks, 8154 genes were affected by glufosinate application in the T plants and 6034 genes in the S plants (Table 5, Figs. 6 and 7). Comparison between treated T and S plants revealed 567 genes that were more repressed or more induced by glufosinate in the treated T plants. Overall, there were 210 upregulated and 357 downregulated genes in the treated T relative to the treated S plants (Figs. 8 and 9). One hundred-five glufosinate-responsive genes were differentially expressed in both treated T (32 genes) and S plants (73 genes) (Fig. 7). In addition, 239 genes that were differentially expressed between treated (52 genes) and non-treated (187 genes) T

plants were more highly repressed or more induced in treated T plants than in treated S plants. Of these 239 genes, the majority were related to biosynthetic process, cellular nitrogen compound and small molecule metabolic processes, response to stress, and oxidoreductase activity (Fig. 9). Among the upregulated genes of this 239-gene subset, 91 were induced by glufosinate in T plants, including genes putatively annotated as NAC transcription factor, *CYP94a1*, and ABC transporter b. The majority of upregulated genes that were differentially expressed between treated T and S plants, and that were also differentially expressed relative to their respective non-treated counterparts, were related to nitrogen compound metabolic processes, oxidoreductase activity, nucleotide binding, and transferase activity. Two genes that were repressed exclusively in the treated T plants relative to the treated S plants were folypolyglutamate synthase and caffeic acid 3-o-methyltransferase (Fig. 7).

The functional classification of selected differentially expressed genes (DEGs) associated with stress response and herbicide metabolism was examined to investigate the pattern of transcriptome regulation that occurred during glufosinate treatment (Fig. 11). Glufosinate treatment triggered the expression of genes related to stress response and xenobiotic detoxification as expected. Some genes associated with photosynthesis, structural stabilization, cell membrane binding, stress response, and detoxification were repressed. Increased expression of non-target site (NTS) genes related to stress response, stress signaling, detoxification, abiotic response, cell structure stabilization, and growth and senescence was observed in treated T plants. Genes that were exclusively induced in treated T plants were annotated to code for transmembrane protein 45b, heat stress transcription factor B, hypersensitive-induced response protein, cytochrome P450 (*Cyp72A219, 94A2, Cyt86b1*-like), transcription factor, ethylene-responsive transcription factor, glutathione S-transferase (*GST*), zinc finger protein *constans-like*

10, and *NAC* transcription factor (Fig 8, Tables 5 and 6). These candidate genes likely play a role in the adaptation of *A. palmeri* to glufosinate and, possibly, also to other herbicides.

Of the 239 genes that were more highly induced or repressed in the treated T plants, 49 were consistently upregulated. These consistently upregulated genes are involved in biosynthetic process, cellular nitrogen compound metabolic process, nucleic acid binding transcription factor activity, oxidoreductase activity, stress response, signal transduction, transferase activity, transmembrane transporter activity, and transport (Table 6 and Fig. 10). A subset of 13 glufosinate-inducible genes which are related to detoxification, stress signaling, and transport are candidate genes involved in conferring some tolerance to glufosinate. These include ABA 8'-hydroxylase, ABC transporter, chitin elicitor receptor kinase, cytochrome P450 72A, cytochrome P450 94A, GST, heat stress transcription factor, heat shock protein 83, ethylene response transcription factor, NAC transcription factor, NAC transcription factor 25, NADH ubiquinone oxidoreductase, and nitronate monooxygenase (NMO) (Table 7). These genes were induced >2-fold in treated T plants relative to treated S plants and non-treated T plants.

Validation of selected genes using qRT-PCR

The expression of seven candidate NTSR genes was measured in two *A. palmeri* accessions using quantitative real-time PCR. Gene expression was similar in the non-treated tolerant and susceptible plants. The 7 genes were induced in T and S plants upon glufosinate treatment. Five genes (*HSP, NMO, ETF, ABC, NAC*) were not differentially expressed between treated tolerant and treated susceptible plants. *GST* was differentially expressed in only one of the treated tolerant plants. On the other hand, *CYP72A219* was expressed eight times higher in all the treated tolerant plants relative to the susceptible ones (Fig. 12).

Discussion

Differential tolerance to glufosinate in *A. palmeri* accessions

The differential response to glufosinate in the 120 *Amaranthus palmeri* accessions demonstrates variation in herbicide efficacy on a weed population (Table 1). Several factors affect glufosinate activity even on a single species; these include temperature, light, relative humidity, time of day, plant size/age, and dose [30, 64-69]. Variation in environmental conditions was minimal in the greenhouse. Variation in plant factors was minimized by maintaining seedlings of the same size. The impact of time of day on glufosinate activity was eliminated by applying the herbicide at about the same time in each repetition of the experiment. Accessions with a few survivors reflect some heterogeneity within the population, as expected of this highly diverse species. Projecting such diversity to field conditions, where all the factors mentioned above can vary, we expect to see a higher frequency of individuals that would escape weed control activities in the field. Many such escapes could have been subjected to sub-lethal doses as a consequence of plant, environmental, and application variables. Controlling these escaped individuals, or adopting a weed management strategy that controls escaping genotypes, is a critical step in mitigating the accumulation of non-target-site genes that could eventually endow resistance to herbicides [70].

Resistance profiling of the 08-Lee-C recalcitrant accession

Herbicides impose strong abiotic stresses to weeds in crop fields, managed turfgrass, gardens, and roadsides. The evolution of resistance in populations of weedy species is an increasing problem worldwide. The recalcitrant *A. palmeri* accession, 08-Lee-C, was resistant to

glyphosate and ALS-inhibiting herbicides, trifloxysulfuron and pyrithiobac (Table 2). The occurrence of resistance to multiple herbicides in 08-Lee-C is not surprising because this field was sprayed with glyphosate and ALS inhibitors for several years. This field was planted with glyphosate-tolerant crops for more than three years and had been exposed to ALS inhibitors in the years prior when the grower was planting conventional soybean. Resistance to glyphosate and ALS inhibitors among *A. palmeri* in Arkansas is widespread [71]. Fomesafen, a PPO herbicide with soil and foliar activity, was first commercialized in the 1960s and had been used by farmers mainly for soybean. The usage of fomesafen, and almost all other herbicides in soybean, dropped when glyphosate-tolerant soybean was introduced in the mid-1990s. Upon the explosion of glyphosate-resistant *Amaranthus* species, soybean farmers reverted to using fomesafen and its use was expanded to cotton to control glyphosate-resistant *Amaranthus* species. The farmer of this field, like many others, had been using only glyphosate to control weeds. Although glufosinate had not been used in this field, some *A. palmeri* individuals displayed differential tolerance to glufosinate. Being an obligate outcrossing species, *A. palmeri* exhibits high genetic diversity, which facilitates its tendency to evolve herbicide resistance. Intensive use of glufosinate in this field, in a manner that allows escapes to produce seed, will accelerate the evolution of resistance through accumulation of multiple low-impact tolerance genes as demonstrated already in some species, including *Lolium rigidum* [72] and *A. palmeri* [73]. Tolerance traits can accumulate and get fixed in the population as selection pressure continues.

Ammonia accumulation in response to glufosinate

Ammonia accumulation is directly related to glufosinate toxicity. Inhibition of glutamine synthetase and ammonia accumulation triggers a cascade of reactions, including inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) enzyme [74] and photosystem electron flow [75], affecting photosynthesis [30, 76] leading to plant death. Ammonia reduces pH gradient across the membrane, which uncouples photophosphorylation [75]. Elevated levels of ammonia accumulated in glufosinate-treated rice and soybean cell cultures [77, 78]. In our study, glufosinate-sensitive plants accumulated 2X more ammonia than the T plants (Fig. 2). Similarly, glufosinate-sensitive *L. perenne* ssp. *multiflorum* from Oregon accumulated 1.6X more ammonia than the resistant population [25]. Increased ammonia level in S plants after glusofinate treatment is the consequence of rapid depletion of functional glutamine synthetase. Reduced ammonia accumulation in T plants indicates the presence of mechanism(s) that reduce the impact of glufosinate on plant function. Such mechanisms could either be reduced binding affinity of glufosinate by target site modification or, mechanisms external to the herbicide-binding site (NTSM) including detoxification and others. The latter applies to the glufosinate-tolerant *A. palmeri* plants.

Glutamine synthetase (*GS2*) copy number

Gene amplification conferring herbicide resistance has been identified in GR weeds such as *A. palmeri, A. tuberculatus, K. scoparia,* and *L. multiflorum* [43, 79, 80]. The GR plants contain multiple copies of *EPSPS*, the target site of glyphosate, which results in increased production of EPSPS enzyme allowing the plant to function normally despite the presence of glyphosate. This mechanism has not been observed with other herbicide target genes either because it is exclusive

to the *EPSPS* regulatory process, or simply because it has not been investigated in other herbicide target genes. Amplification of *GS2*, in glufosinate-resistant weeds, is not yet reported. However, a 4- to 11-fold amplification of *GS2* in alfalfa cell culture lines resulted in increased GS enzyme production, endowing resistance to glufosinate [81]. In our study, S and T *A. palmeri* had similar copies (1-3) of *GS2* indicating that tolerance to glufosinate was not due to *GS2* amplification (Fig. 3). This was supported by the fact that *GS2* transcripts were not different between S and T plants.

Glutamine synthetase (*GS2*) gene sequence

A rare individual in a population may harbor a mutation in the herbicide-binding site that can alter the folding structure of the protein, resulting in reduced binding affinity of the herbicide. Resistance to ALS-inhibiting herbicides in weeds, in most cases, is due to mutation(s) in one or more of the binding domains in the ALS enzyme [82]. The higher frequency of SNPs in *GS2* of T plants could predispose such individuals in the population to accumulate nonsynonymous nucleotide substitutions. However, genetic polymorphisms may not always get translated to protein polymorphisms, and only certain amino acid mutations will result in herbicide resistance [82]. In the current study, most of the polymorphisms observed in the nucleotide sequence of *GS2* in T plants were synonymous. Although seven amino acid substitutions in the upstream region were detected in one of the alleles of the T biotype, these substitutions also occur in glufosinate-sensitive *A. viridis* and is in a region outside of the substrate-binding domain (Fig. 4). A Tyr_8Asn substitution was detected in the two GS2 alleles of the T biotype. Asparagine and tyrosine are both polar, uncharged amino acids, hence Tyr_8Asn substitution may not alter the physiological and physicochemical stability of the plastid GS enzyme. The presence of this

trivial amino acid substitution in glufosinate-tolerant plants as well as similar *GS2* copies as that of the S plants suggests that non-target-site tolerance factors are involved. Transcriptome analysis could inform us on differential tolerance mechanisms. A glufosinate-resistant *L. perenne* ssp. *multiflorum* from Oregon, USA, which showed similar level of ammonia accumulation to the tolerant *A. palmeri* in our study, harbors a $Asn_{171}Asp$ *m*utation (GAC TO AAC) in the *GS2* gene which confers resistance to glufosinate [25].

Tolerance level to glufosinate in 08-Lee-C and C1

After one cycle of selection, the GR_{50} for C1 increased from 1.44-fold to 2.80-fold relative to SS, reflecting increased frequency of tolerant plants in C1 (Table 3). Although a low frequency (<10%) of the plants survived exposure to glufosinate, the increase in GR_{50} after one cycle of glufosinate selection is indicative that the population has become less sensitive to glufosinate after one cycle of purifying selection. Because glufosinate had not been used in the field, the frequency of survivors was low, and the tolerance level was only 1.44 to 2.80-fold, it is likely that these plant variants have low-level, non-target site resistance. We are possibly capturing an early phase of herbicide resistance evolution. Should the population continue to be under selection pressure from glufosinate, the probability of the population acquiring additional adaptive alleles and expressing resistance to field use rate of glufosinate would increase.

Transcriptome of *A. palmeri* and candidate NTS genes involved in glufosinate tolerance

A reference cDNA transcriptome consisting of 72,794 sequences was assembled for *A. palmeri* (BioProject PRJNA390774, TaxId 107608). The transcriptome of *A. hypochondriacus*

had 57,658 assembled sequences [36]. Our data demonstrated broad effects of glufosinate on several metabolic pathways, as expected of a herbicidal compound, including nitrogen assimilation and metabolism similar to what is reported in *Arabidopsis* [83]. One of the apparent functional categories to which glufosinate-responsive genes belong is protein families known to participate in metabolism, stress response, and defense, with the majority of these genes potentially associated with abiotic stress response signaling and chemical detoxification pathways (Figs. 10 and 11). Stress response genes are inducible by many other herbicides or stress factors. Generally, abiotic stress such as herbicide, salinity, and drought modulates the expression of genes that are involved in signaling cascades and in transcriptional control [84], genes that code for proteins involved in membrane protection [85], and genes that are involved in water and ion uptake and transport [86, 87]. These stress-regulated genes are activated to counteract the stress effects, maintain homeostasis, and adapt. Cell membrane receptor-kinases, stress signaling genes, detoxification-related genes, and antioxidants were activated upon glufosinate treatment in both S and T plants (Fig. 11). Peroxidase and superoxide dismutase, for example, were upregulated to help counteract the oxidative stress caused by lipid peroxidation resulting from glufosinate treatment. This indicates that plants undergo extensive transcriptional adjustment in response to herbicide-induced stress. Activation of herbicide-stress-response genes is hypothesized to be initiated by a herbicide sensor, which triggers the activation of regulator genes, which causes a cascade of reactions to either detoxify the herbicide or protect the plant from herbicide-mediated stress [35].

Two genes, folypolyglutamate synthase and caffeic acid 3-o-methyltransferase, were repressed in the treated T relative to S plants, but were not differentially expressed in other pairwise comparisons. Both are involved in one-carbon transfer and phenylpropanoid

biosynthesis [88, 89]. The phenylpropanoid pathway serves as a rich source of metabolites in plants, especially for lignin biosynthesis and the production of flavonoids, coumarins, hydrocinnamic acid conjugate, cutins and lignins [90, 91]. Phenylpropanoids are involved in plant defense, structural support and survival [91, 92]. Repression of genes involved in phenylpropanoid biosynthesis indicate less allocation of carbon resources to these plant products in the T plants after glufosinate treatment compared to the S plants, indicating carbon allocation to other intermediates is more critical for survival under herbicide stress.

Thirteen candidate genes were identified which included ABA 8'-hydroxylase, ABC transporter (ABC), chitin elicitor receptor kinase (CERK1), cytochrome P450 72A (CYP72A219), cytochrome P450 94A, glutathione S-transferase (GST), heat stress transcription factor, NAC transcription factor, NAC transcriptor 25, ethylene-response transcription factor (ETF), heat shock protein 83 (HSP), NADH ubiquinone oxidoreductase and nitronate monooxygenase (NMO) (Table 6). The expression of these candidate genes was induced by glufosinate treatment. Delye [35] proposed a model of NTS resistance mechanism in which herbicide stress triggers the expression of 'protectors' and 'regulators', as well as epigenetic modifiers which enable the plant to survive herbicide stress. Protector genes include cytochrome P450, oxidase, peroxidases, esterases, hydrolases, glutathione S-transferases and transporters, which play roles in reducing the efficacy of herbicide by detoxification. 'Regulator' genes are involved in transcriptional, post-transcriptional, and post-transductional control such as transcription factors, micro-RNAs, and kinases [35]. It is noteworthy that the candidate NTS genes could act as either 'protector' or 'regulator' based on their functions. Cytochrome P450, GST, NADH ubiquinone oxidoreductase, and ABC transporter proteins have roles in pesticide detoxification [93-98]. Chitin elicitor receptor kinase, ABA 8'-hydroxylase, ethylene-response

transcription factor, heat stress transcription factor, and NAC transcription factor are involved in stress response signaling and regulation [35, 89, 99-102].

Of the seven candidate genes subjected to qRT-PCR validation experiment, only two genes, (cytochrome P450 *CYP72A219* and *GST*) were associated with tolerance to glufosinate (Fig. 12). The induction of *GST* and *CYP72A219* suggests that T plants are able to deactivate glufosinate to some extent. Induction of cytochrome P450 and GST facilitate the conversion of the herbicide into a less toxic metabolite. The biochemical role of cytochrome P450-mediated herbicide metabolism has been well established in herbicide-resistant weed species. Plant cytochrome P450s facilitate the detoxification of toxic xenobiotics by catalyzing oxygen- and NADPH-dependent mono-oxygenation reactions which convert herbicide into a more hydrophilic metabolite [103]. Hundreds of P450 genes exist in higher plants. For example, *Arabidopsis thaliana* and *Oryza sativa* possess 272 and 458 putative P450 genes, respectively [104]. RNA-Seq transcriptome analysis of *L. rigidum* identified CYP72A genes to be involved in metabolic resistance to diclofop [37]. Non-target-site ACCase and ALS resistance in *Alopecurus myosuroides* [95, 96], *Stellaria media* [105], *Lolium* [96, 106-108], *Sinapis arvensis* [109], *Echinochloa phyllopogon* [110], and *Digitaria sanguinalis* [111] were reported previously to be facilitated by cytochrome P450 enzymes. Upregulation of *CYP72A* and *CYP94A* was reported in a multiple-herbicide-resistant *E. phyllopogon* population [112]. Similarly, *CYP94A1*, a plant cytochrome P450-catalyzing fatty acid omega hydroxylase, was induced by chemical stress in *Vicia sativa* and by bentazon treatment in soybean [113, 114]. Some cytochrome P450 genes in the CYP71A family were also demonstrated to be involved directly in herbicide metabolism in crops, such as *O. sativa CYP71A31* and Z*ea mays CYP71A2*8 [115, 116].

The involvement of glutathione *S*-transferases (GSTs) in herbicide resistance is reported in several weed species. Glutathione *S*-transferases are ubiquitous enzymes that catalyze the conjugation of harmful xenobiotics to reduced glutathione, facilitating their metabolism, sequestration or removal [117]. The primary factor for atrazine selectivity in corn is the activity of a soluble GST, which detoxifies atrazine by forming an atrazine-glutathione conjugate [118]. In a recent transcriptome study, increased expression of *GST* is associated with diclofop resistance in *L. rigidum* and nicosulfuron tolerance in *Z. mays* [37, 116]. GST also functions as an antioxidant, protecting plants from herbicide-mediated oxidative stress by scavenging reactive oxygen species [119]. Increased expression of glutathione transferase gene (*AmGSTF1*) in multiple-resistant *A. myosuroides* led to accumulation of flavonoids which protects the plant from herbicide injury [120]. It has been reported that glutathione transferase orchestrate tolerance to abiotic stress through their ability to regulate redox signaling pathways that activate defense genes [121]. In glufosinate-tolerant *A. palmeri*, GST is possibly involved in converting glufosinate into a less toxic metabolite following possible minimal phase I detoxification by CYP72A219 as well as in protecting the plants against oxidative stress and lipid peroxidation from glufosinate phytoxicity.

Other candidate genes were identified by RNASeq, but were not differentially expressed in the validation experiment, including as the ABC transporter, NAC transcription factor, NMO, HSP, and ethylene transcription factor. Although these might not be involved in conferring some level of tolerance to glufosinate, their involvement in detoxification of toxic xenobiotic compounds and in stress response have been reported. For example, NMO in *A. thaliana* is associated with detoxification of the allelochemical benzoxazolin [122]. Increased expression of NADH ubiquinone oxidoreductase and induction of P450 genes were involved in resistance to

pyriproxyfen insecticide in *Bemisia tabaci* [123]. Induction of heat shock proteins is associated with drought and oxidative stress in *Brassica juncea* [124] and *A. thaliana* [125], respectively. Plant ABC transporters have been associated with the movement of herbicide conjugates [126, 127]. Modifications of ABC transporters have been suspected in some cases of weed resistance to glyphosate or paraquat [128, 129]. Tolerance of *Arabidopsis thaliana* to paraquat is endowed by a mutation in the plasma membrane-localized ABC transporter, which resulted in reduced herbicide uptake in plant cells [130]. NAC transcription factors and ethylene-response transcription factors play an important role in the regulation of the transcriptional reprogramming associated with plant stress response such as cold, drought, and salinity [131-134].

Diversity in gene expression and regulation is an important factor driving herbicide resistance evolution [135]. As gene expression regulation also involves post-transcriptional and post-translational controls, protein expression of the identified genes may need to be further investigated. Because of genetic diversity, plants have the potential to overcome herbicide stress through a concerted effort of multiple genes. Plants with low-level tolerance showed greater induction of abiotic stress-protection- and detoxification-related genes than S plants. Thus, survival from glufosinate treatment is facilitated by stress-protection/stress-adaptation genes. Differential expression of stress-protection genes in a population can enable some individuals to survive herbicide application. Tolerance-related genes can get fixed in a population upon the exertion of sustained selection pressure. Selection pressure coupled with genetic diversity drives evolutionary processes leading to herbicide resistance.

A low frequency of a low-level presumed tolerance in the phenotype was observed across a two-stage screening of survivors from a recalcitrant, segregating population. The surviving individuals were described as tolerant because there was no record of their exposure to

glufosinate in this field. However, a three-year record, which is what growers can provide generally, is insufficient to assert that the gene regulation demonstrated here is truly ancestral and that it would have been expressed in the total absence of glufosinate selection. *Amaranthus palmeri* is a prolific dioecious plant. Seed could have been transported to the field from elsewhere where glufosinate has been used. Similarly, pollen from other fields with a history of glufosinate use, could have blown through the field and fertilized female plants. Differential gene expression of *CYP72A219* and *GST* and the presence of surviving progeny in intentionally intercrossed populations show that genetic mechanisms exist for the evolution of low-level, or potentially incipient, resistance to glufosinate in some *A. palmeri* populations.

Natural variation in sensitivity to herbicides among individuals exists in *A. palmeri* population. The inherent ability of *A. palmeri* to tolerate glufosinate, or any other herbicides, due to non-target mechanism(s) will impact the dynamics and evolution of herbicide-resistant populations. The frequency of glufosinate-tolerant biotypes in a population will increase with continued herbicide exposure. Non-target-genes could accumulate and get fixed in the population leading to evolution of resistance. Vigilance will be required to detect elevated glufosinate tolerance, especially because it is multi-genic, impossible to eliminate from populations, and eventually might confer resistance to more than one class of herbicide chemistries and abiotic stresses. If proactive measures are ignored, it is just a matter of time that glufosinate-resistant *A. palmeri* populations will evolve and cause formidable task in weed management.

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Table 1. Cluster analysis of *A. palmeri* accessions treated with glufosinate at 0.55 kg ha^{-1}.

Cluster	Number of accessions	Mortality (%)			Mean frequency of plants at different levels of injury (%)					
		Mean	Min	Max	0-10% injury	11-30% injury	31-60% injury	61-80% injury	81-99% injury	100% injury
1	88	100	100	100	0	0	0	0	0	100
2	28	98	94	99	0	0	1	1	0	98
3	4	92	88	97	0	1	3	4	0	92

Table 2. Response of *A. palmeri* (08-Lee-C) to foliar-applied herbicides.

Herbicide	Mortality (%)[a]	Mode of action[b]
Dicamba	99	Synthetic auxin
Fomesafen	98	PPO inhibitor
Glufosinate	93	Glutamine synthetase inhibitor
Glyphosate	61	EPSP synthase inhibitor
Pyrithiobac	21	ALS inhibitor
Trifloxysulfuron	25	ALS inhibitor

[a]Uniform-sized plants (7.5-9 cm tall) were sprayed with dicamba (280 g ha^{-1}), fomesafen (264 g ha^{-1}) glufosinate (0.55 kg ha^{-1}), glyphosate (870 g ha^{-1}), pyrithiobac (73 g ha^{-1}), and trifloxysulfuron (8 g ha^{-1}). Mortality was recorded 21 d after herbicide application.
[b]PPO- protoporphyrinogen oxidase, EPSP- enolpyruvyl shikimate-3-phosphate, ALS- acetolactate synthase

Table 3. Glufosinate dose required to reduce growth by 50% (GR_{50}) in *A. palmeri* 08-Lee-C, C1 and SS accessions.

Accession	**GR_{50}**	**Confidence Intervals[a]**	**T/S[b]**
	kg ai ha^{-1}		
08-Lee-C	0.110	0.097 - 0.123	1.44
C1	0.214	0.184 - 0.244	2.80
SS[c]	0.076	0.064 - 0.088	

[a] 95% confidence intervals.
[b] Tolerance levels (T/S) calculated using the GR_{50} of the tolerant accession relative to the susceptible standard.
[c] Herbicide-susceptible standard accession.

Table 4. Summary of statistics for transcriptome assembly.

	Reads (n)	Bases (Mb)	Average length (bp)
Illumina raw reads	1,667,277, 670	8409.7	125
Assembled contigs	72,780	49.15	675
Annotated sequences (blastX)	65,282	-	-
Sequences assigned with GO terms	33,294	-	-

Table 5. Differentially expressed genes putatively involved in differential tolerance to glufosinate in *Amaranthus palmeri*.

Level of gene expression	**Number of differentially expressed genes**							
	TWO vs SWO		SWT vs SWO		TWT vs TWO		TWT vs SWT	
	Repressed	Induced	Repressed	Induced	Repressed	Induced	Repressed	Induced
>1-2	2	0	589	353	1277	1048	0	0
>2-3	43	26	1228	994	1846	1229	33	11
>3-4	58	72	662	677	756	679	94	65
>4-5	27	64	328	389	299	351	74	41
>5-6	13	36	109	218	125	182	63	31
>6-7	4	22	72	146	55	135	23	22
>7-10	8	51	45	175	37	113	47	33
>10	3	9	10	39	1	21	23	7
Total	158	280	3043	2991	4396	3758	357	210
	438		6034		8254		567	

[a]TWO vs SWO: non-treated tolerant (T) relative to non-treated susceptible (S) plants; SWT vs SWO: treated S relative to non-treated S plants; TWT vs TWO: treated T relative to non-treated T plants; TWT vs SWT: treated T relative to treated S plants

Table 6. Upregulated genes in glufosinate-treated and non-treated tolerant (T) plants, and in glufosinate-treated T relative to treated susceptible (S) plants, assigned with Gene Ontology molecular function and biological process related to metabolism and signaling pathways.

GO function	Contig	Gene annotation	Fold change	
			TWT_n/TWO_n	TWT_n/SWT_n
biosynthetic process	Pa27529	50S ribosomal protein chloroplastic	**8.47**	**9.02**
biosynthetic process	Pa29824	60s ribosomal protein l13a-2	**7.60**	6.25
biosynthetic process	Pa38623	phenazine biosynthesis-like domain-containing protein 1 isoform x2	**6.93**	3.92
biosynthetic process, cellular nitrogen compound metabolic process, nucleic acid binding transcription factor activity	Pa65724	cyclic dof factor 1-like	**3.67**	4.46
biosynthetic process, small molecule metabolic process	Pa35601	phosphoribosylaminoimidazole chloroplastic-like	**9.24**	11.22
cellular amino acid metabolic process, biosynthetic process, small molecule metabolic process	Pa17844	shikimate chloroplastic	**2.49**	4.41
cellular amino acid metabolic process, secondary metabolic process	Pa39917	3-isopropylmalate dehydratase -like protein	**4.86**	5.79
cellular amino acid metabolic process, small molecule metabolic process	Pa60555	probable low-specificity l-threonine aldolase 1	**7.04**	5.40
cellular nitrogen compound metabolic process	Pa52820	putative polyprotein	**2.55**	4.88
cellular nitrogen compound metabolic process	Pa63676	CTP synthase	**1.64**	3.18
cellular nitrogen compound metabolic process	Pa71553	gag-pol polyprotein	**5.93**	7.09
cellular nitrogen compound metabolic process	Pa8879	zinc finger bed domain-containing protein ricesleeper 1-like	**7.63**	9.05
cellular nitrogen compound metabolic process, biosynthetic process, signal transduction	Pa63868	two-component response regulator arr9 isoform x1	**2.20**	3.43
cellular nitrogen compound metabolic process, biosynthetic process	Pa37812	NAC transcription factor	**8.25**	7.80
cellular nitrogen compound metabolic process, biosynthetic process, signal transduction	Pa51700	two-component response regulator arr5-like isoform x2	**1.62**	2.58
cellular nitrogen compound metabolic process, response to stress, biosynthetic process	Pa37809	heat stress transcription factor b-2b-like	**3.01**	2.88
cellular nitrogen compound metabolic process, response to stress, biosynthetic process, signal transduction	Pa13900	RNA polymerase ii c-terminal domain phosphatase-like 1	**3.52**	3.83
hydrolase activity, acting on glycosyl bonds	Pa26833	PREDICTED: alpha-glucosidase-like [*Beta vulgaris* subsp. vulgaris]	**6.20**	4.47

Table 6 (Cont.)

GO function	Contig	Gene annotation	Fold change	
			TWT_n/TWO_n	TWT_n/SWT_n
hydrolase activity, acting on glycosyl bonds	Pa42036	alkaline neutral invertase cinv2-like	**3.40**	3.15
hydrolase activity, acting on glycosyl bonds, response to stress	Pa69030	beta-amylase chloroplastic	**3.94**	5.41
nucleic acid binding transcription factor activity, biosynthetic process, signal transduction	Pa49594	auxin-responsive protein iaa29	**4.11**	4.51
nucleic acid binding transcription factor activity, cellular nitrogen compound metabolic process, biosynthetic process	Pa47424	NAC transcription factor 25-like	**1.98**	3.34
nucleic acid binding transcription factor activity, cellular nitrogen compound metabolic process, response to stress, biosynthetic process, signal transduction	Pa38292	ethylene-responsive transcription factor abr1	**5.24**	4.92
oxidoreductase activity	Pa10467	cytochrome P450 cyp72A219-like	**8.56**	4.42
oxidoreductase activity	Pa40402	internal alternative NAD H-ubiquinone oxidoreductase mitochondrial	**5.22**	3.49
oxidoreductase activity	Pa45867	nitronate monooxygenase	**6.26**	4.28
oxidoreductase activity	Pa51578	-dopa dioxygenase extradiol-like protein	**6.44**	5.06
oxidoreductase activity	Pa56011	short-chain type dehydrogenase reductase-like	**7.89**	3.99
oxidoreductase activity	Pa60473	cytochrome P450 94a1-like	**2.90**	3.34
oxidoreductase activity, cellular amino acid & metabolic process, cellular amino acid metabolic process, homeostatic process, oxidoreductase activity	Pa10326	5 -adenylylsulfate reductase chloroplastic- partial	**3.47**	4.04
oxidoreductase activity, small molecule metabolic process	Pa44392	abscisic acid 8 -hydroxylase 2	4.153201	4.072155
response to stress, immune system response	Pa52955	macpf domain-containing protein at1g14780	1.801976	3.089814
response to stress, immune system response	Pa62900	heat shock protein 83	4.420622	4.392136
response to stress, signal transduction, immune system process	Pa42133	chitin elicitor receptor kinase 1-like	3.354966	3.353194
response to stress, signal transduction, immune system process	Pa69811	receptor-like protein kinase at3g47110	3.401606	6.459389
response to stress, transport, transmembrane transport	Pa53135	mitochondrial phosphate carrier protein mitochondrial-like	11.42314	4.210532
signal transduction	Pa60381	receptor-like serine threonine-protein kinase sd1-8 isoform x1	5.532361	3.68854
signal transduction	Pa63442	PREDICTED: uncharacterized protein LOC104887975	3.319583	5.558233

Table 6. (Cont.)

GO function	Contig	Gene annotation	Fold change	
			TWT_n/TWO_n	TWT_n/SWT_n
transferase activity, transferring acyl groups	Pa67068	uncharacterized acetyltransferase at3g50280-like	10.27052	4.376485
transferase activity, transferring alkyl or aryl (other than methyl) groups	Pa19271	glutathione s-transferase-like protein	10.47473	6.70153
transferase activity, transferring glycosyl groups, biosynthetic process	Pa49933	7-deoxyloganetin glucosyltransferase-like	6.037006	4.239084
transferase activity, transferring glycosyl groups, response to stress, bioysnthetic process, small molecule metabolic process, cell wall organization or biogenesis	Pa57353	gdp-l-galactose phosphorylase 2-like	5.457352	5.232313
transmembrane transporter activity	Pa14919	peroxisomal nicotinamide adenine dinucleotide carrier-like	1.675916	4.465376
transmembrane transporter activity	Pa21499	calcium-transporting atpase plasma membrane-type	4.459466	3.358566
transmembrane transporter activity	Pa35784	mate efflux family protein 9-like	3.45508	3.260395
transmembrane transporter activity	Pa63432	anoctamin-like protein at1g73020	3.788903	3.221265
transmembrane transporter activity, cellular nitrogen compound metabolic process, transport, small molecule metabolic process	Pa63215	ABC transporter b family member 2-like	4.662064	6.567808
transport	Pa60553	outer envelope protein mitochondrial	3.146434	7.2662

[a]TWT = treated T plants
TWO = non-treated T plants
SWT = treated S plants

Table 7. Candidate non-target genes, identified by RNA-Seq analysis, that are potentially involved in conferring differential tolerance to glufosinate in *A. palmeri.*

Contig	Gene annotation	Fold change[a]		Function
		TWT_n/TWO_n	TWT_n/SWT_n	
19271	Glutathione S-transferase (*GST*)	10.47	6.7	Detoxification
10467	Cytochrome P450 CYP72A219	8.55	4.42	Heme-thiolate monoxygenase; detoxification
37812	NAC transcription factor	8.24	7.8	Transcription regulator in plant stress response
38292	Ethylene-response transcription factor abr1	5.24	4.91	ABA signaling pathway in response to stress response
40402	NAD H-ubiquinone oxidoreductase	5.21	3.49	Detoxification
63215	ABC transporter b family member 2	4.66	6.56	Transmembrane transport
62900	Heat shock protein 83	4.31	8.2	Molecular chaperone; stress signaling
44392	ABA 8'-hydroxylase	4.15	4.07	ABA catabolism
45867	Nitronate monooxygenase (*NMO*)	6.26	4.27	Detoxification
42133	Chitin elicitor receptor kinase 1 (*CERK1*)	3.35	3.35	Cell surface receptor toward biotic and abiotic stresses
37809	Heat stress transcription factor b	3.01	2.87	Transcription regulator for heat shock proteins; stress signaling
60473	Cytochrome P450 94a1	2.9	3.33	Detoxification
47424	NAC transcription factor 25-like	1.97	3.34	Abiotic stress response

[a]TWT = treated tolerant plants
TWO = non-treated tolerant plants
SWT = treated sensitive plants

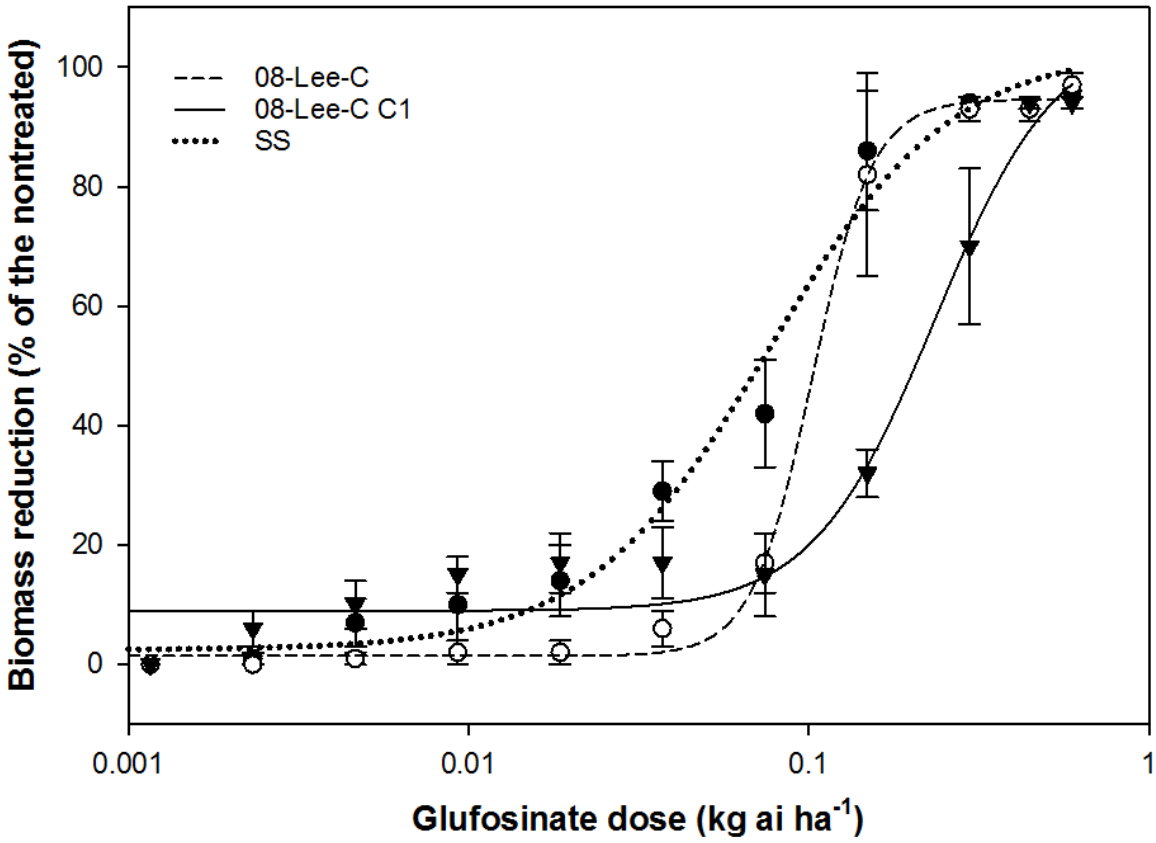

Figure 1. Shoot biomass reduction (%) of 08-Lee-C, C1, and SS *A. palmeri* accessions, 21 days after glufosinate treatment. Data were best described with nonlinear, sigmoidal, four-parameter logistic regression function.

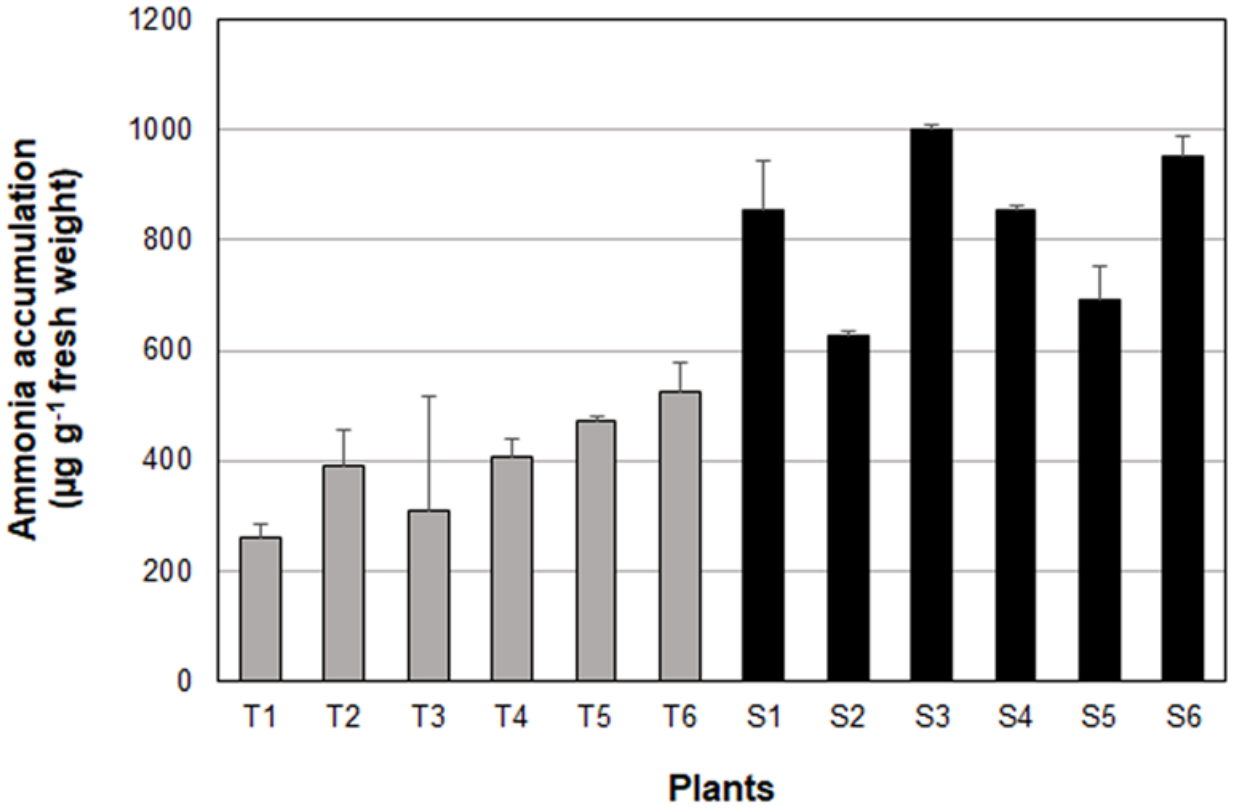

Figure 2. Ammonia content in glufosinate-tolerant (T) and –susceptible (S) *A. palmeri*.Error bars represent standard error. White bars = S plants; gray bars = T plants.

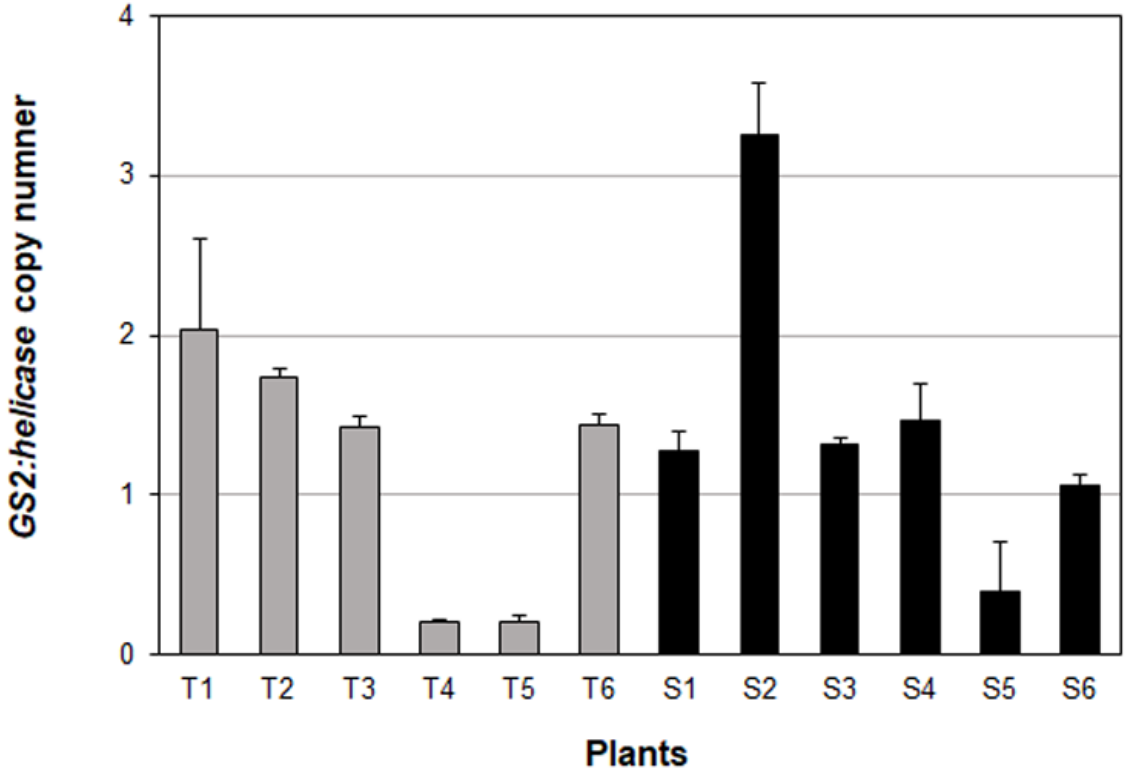

Figure 3. Relative copy number of A. palmeri GS2 in glufosinate-susceptible (S) and -tolerant (T) plants. Error bars represent standard deviation of the mean. Gray bars = T plants; black bars = T plant.

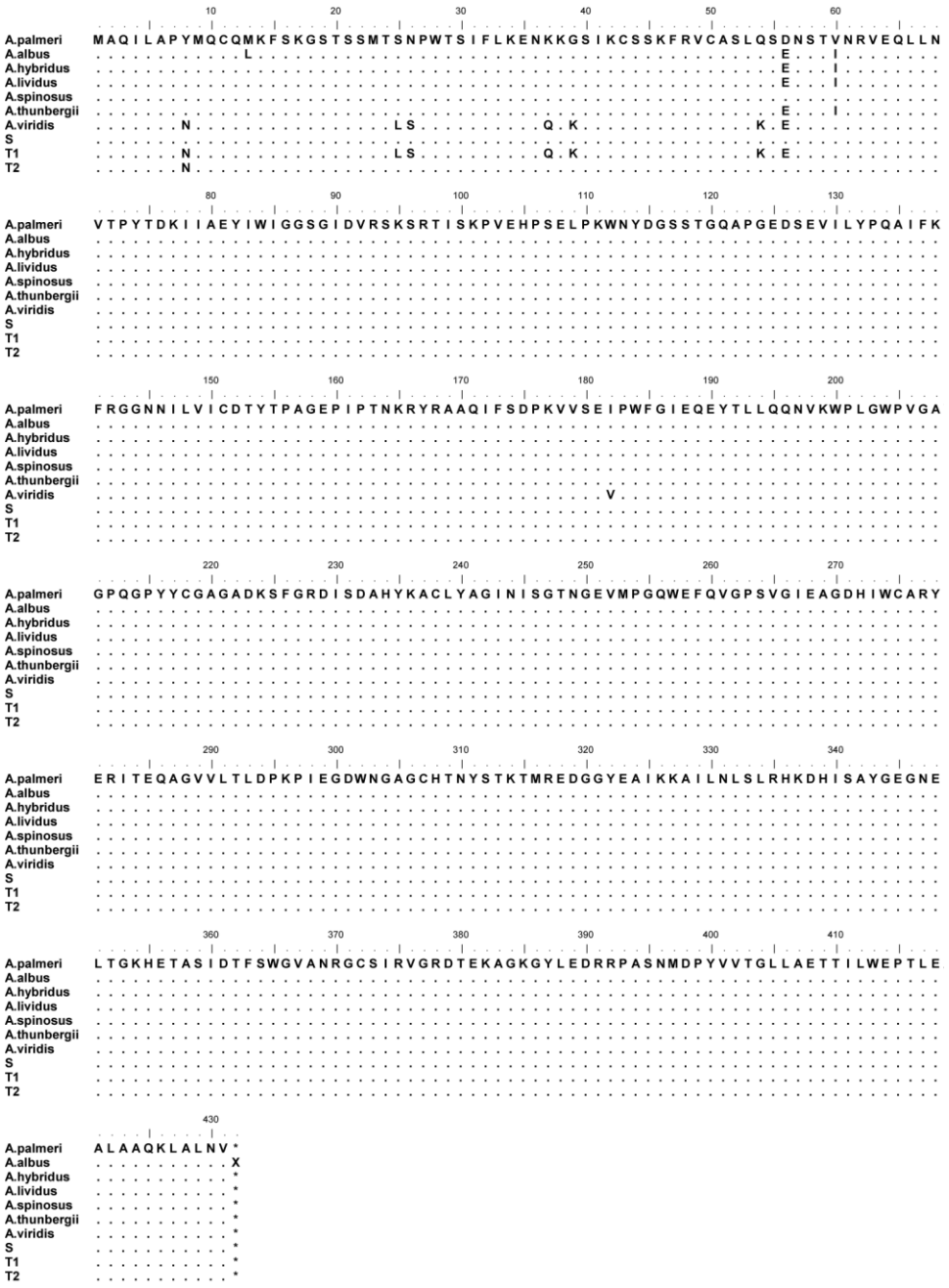

Figure 4. Multiple alignment of the plastidic glutamine synthetase (GS2) amino acid sequences in *Amaranthus*. *A. palmeri* (reference *A. palmeri*), T1 and T2 = GS2 alleles of glufosinate-tolerant *A. palmeri* biotype, S = GS2 sequence of glufosinate-susceptible *A. palmeri* biotype.

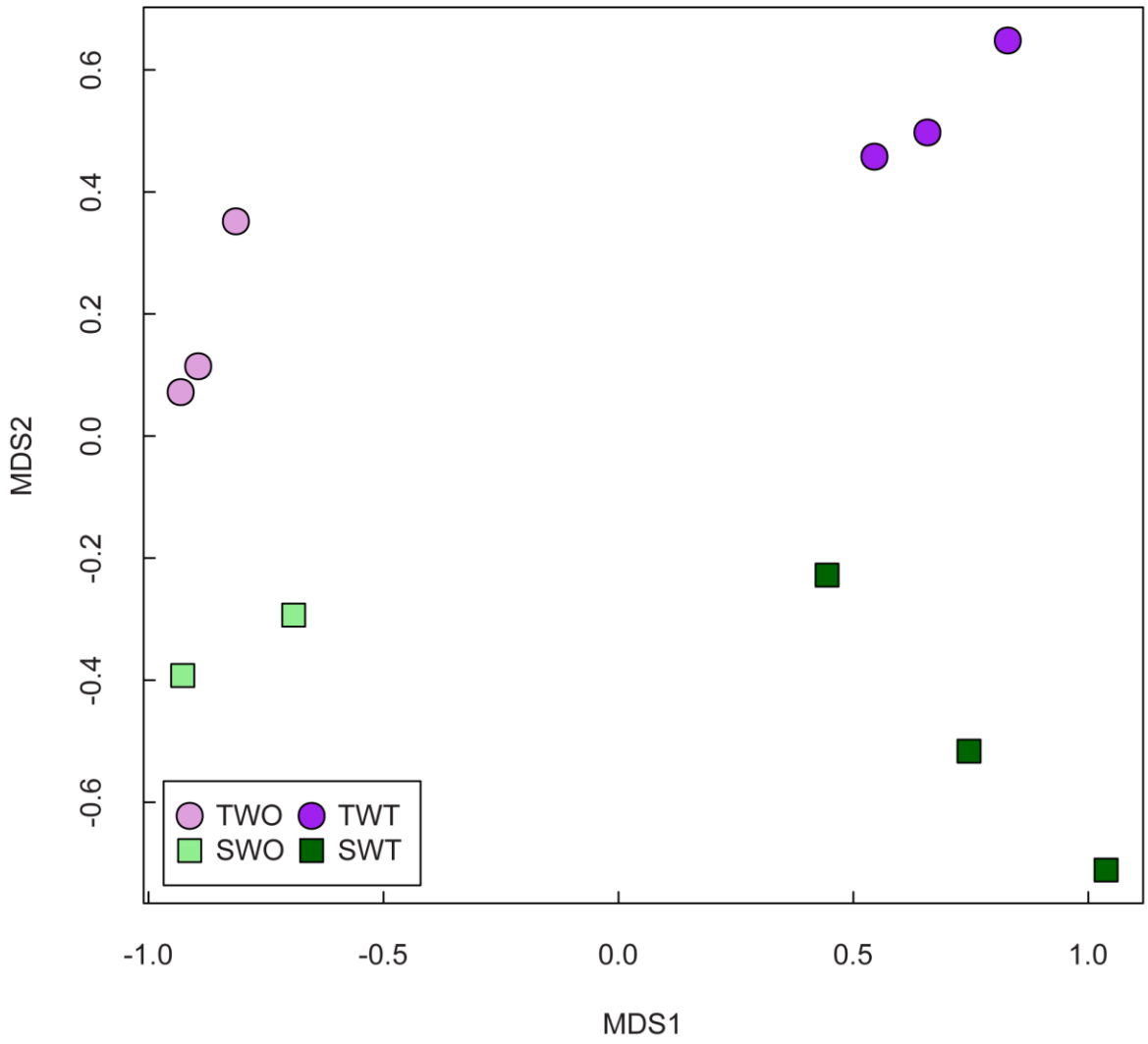

Figure 5. Multidimensional scaling (MDS) plot showing the relationship between sample types.TWO = non-treated tolerant, TWT= treated tolerant, SWO = non-treated susceptible, SWT = treated susceptible.

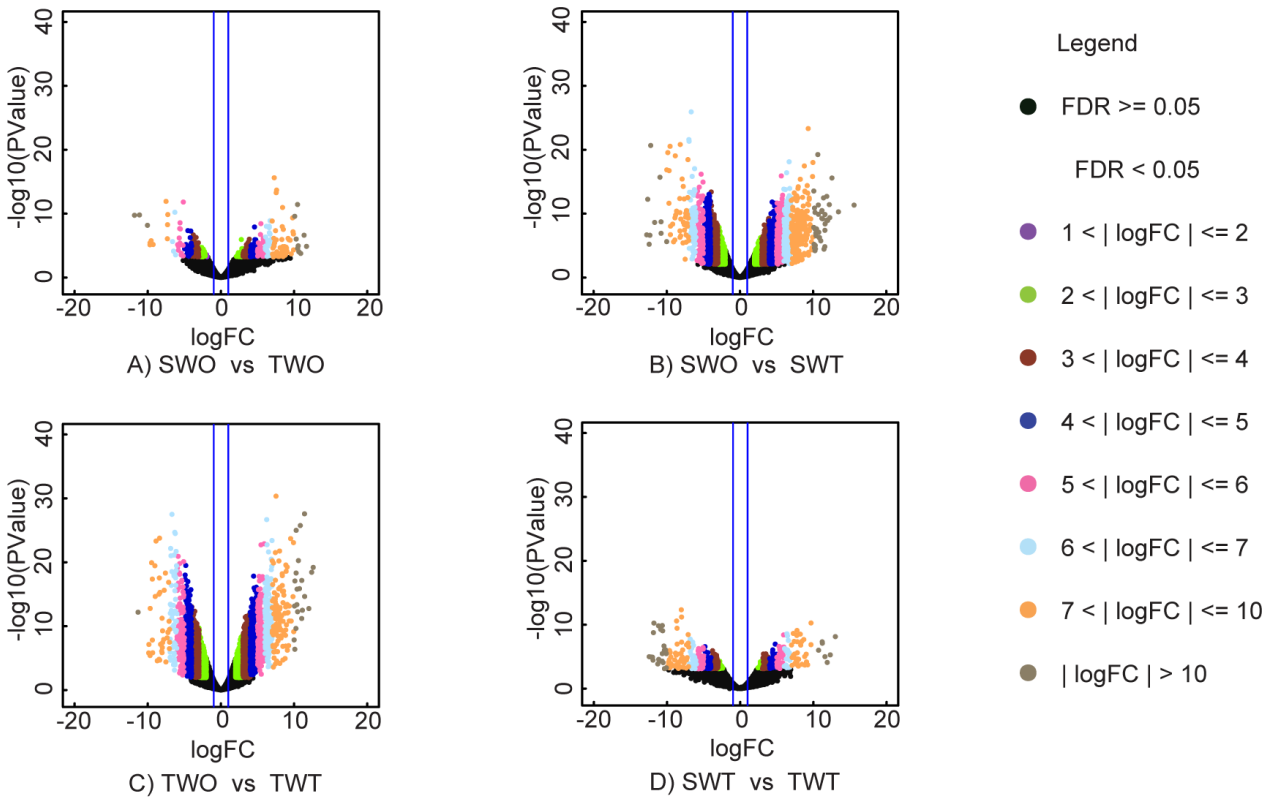

Figure 6. Volcano plots depicting differential gene expression between treatments. A) Treated susceptible (S) relative to non-treated S plants (SWT vs SWO), B) Treated tolerant (T) relative to treated S plants (TWT vs SWT), C) treated T relative to non-treated T plants (TWT vs TWO), and D) treated T plants relative to non-treated S plants (TWO vs SWO). The x-axis shows the log fold change or relative abundance. The P value (-log base 10) for differential gene expression is plotted on the y axis. Black dots represent genes that did not change in expression; colored dots on the left indicate genes with significantly downregulated expression; colored dots on the right indicates genes with significantly upregulated expression.

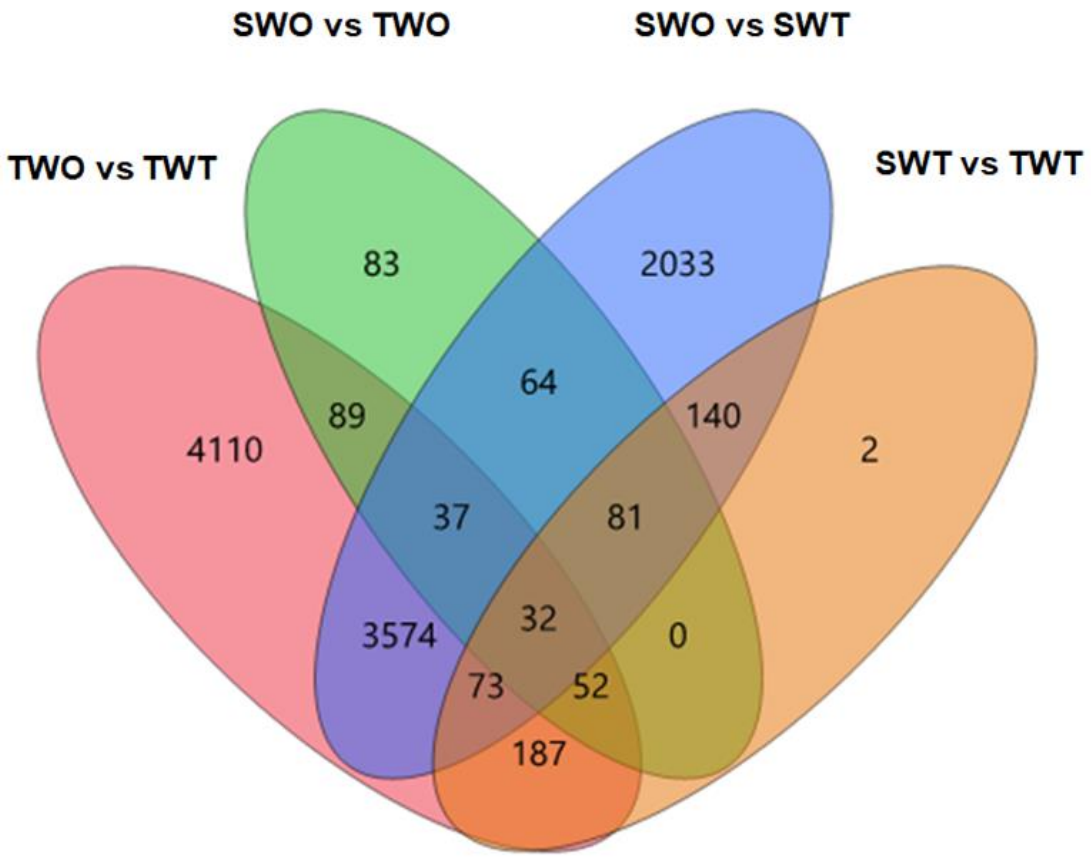

Figure 7. The number of differentially expressed genes common or specific to treated and non-treated T and S plants.A 4-way Venn diagram depicting the distribution of differentially expressed genes across all pairwise comparisons. The number within each shaded area is the number of differentially expressed genes common between treatments.

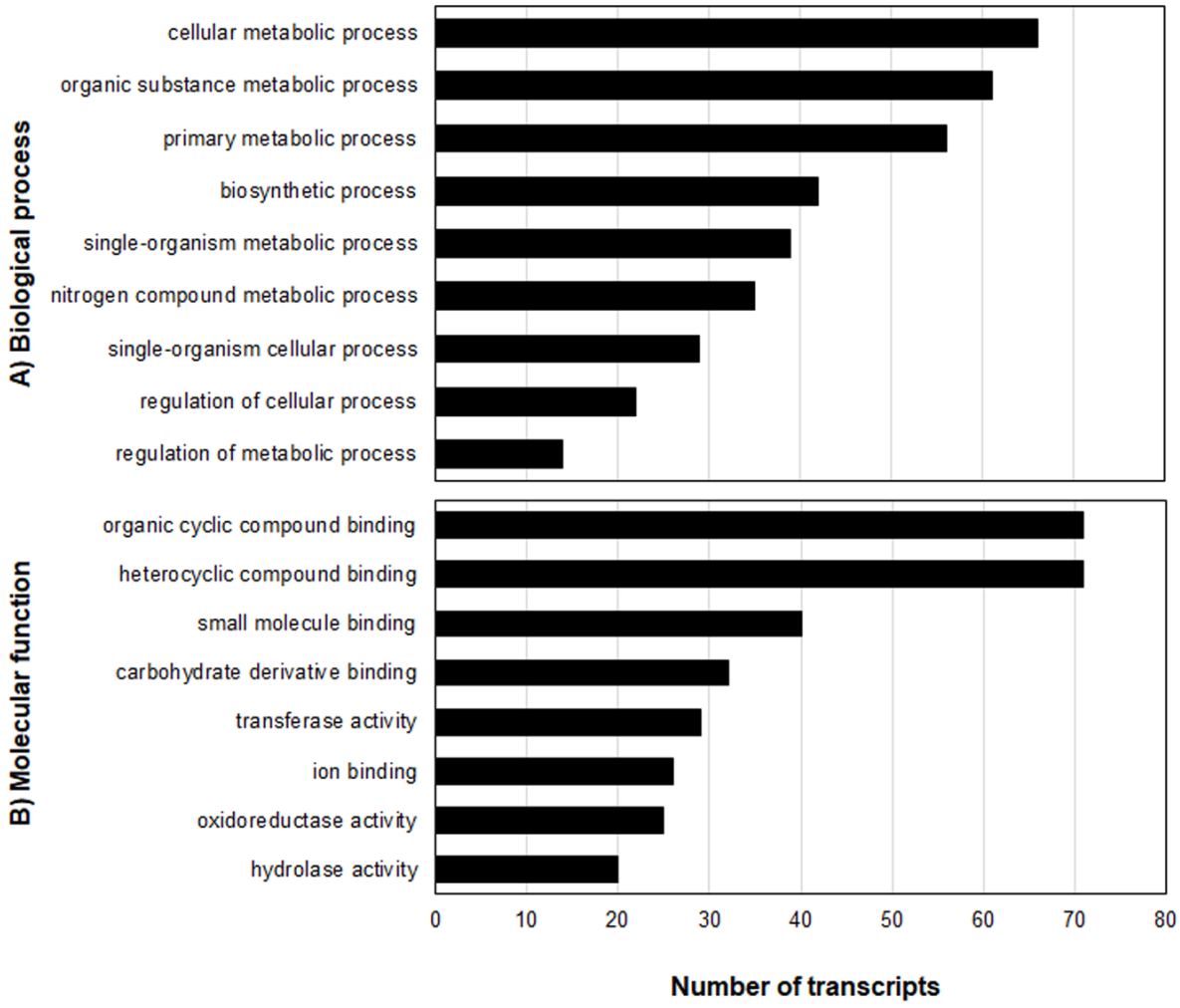

Figure 8. Biological processes (A) and molecular functions (B) of upregulated genes in treated T relative to treated S plants.

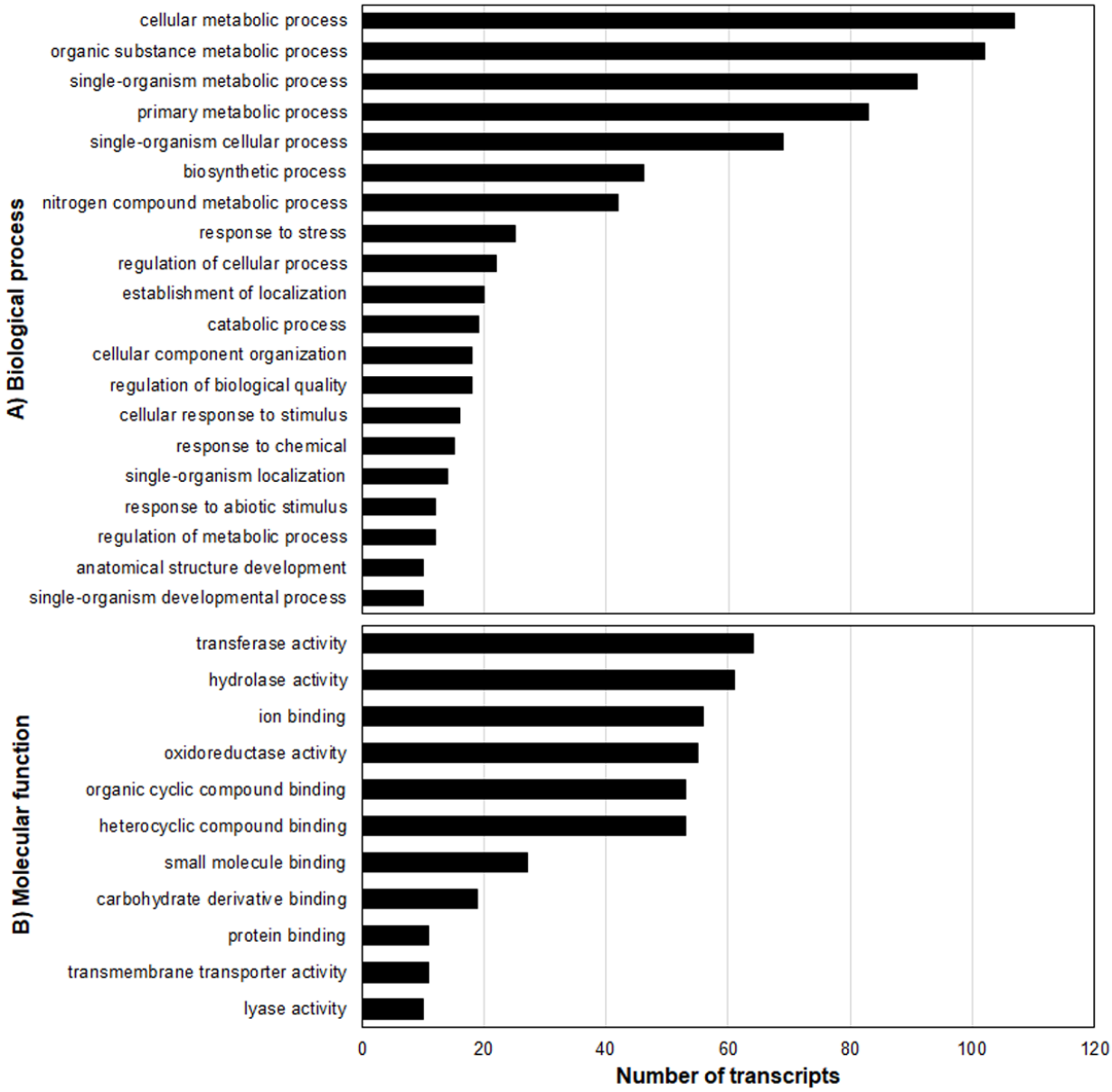

Figure 9. Biological processes (A) and molecular functions (B) of downregulated genes in treated T relative to treated S plants.

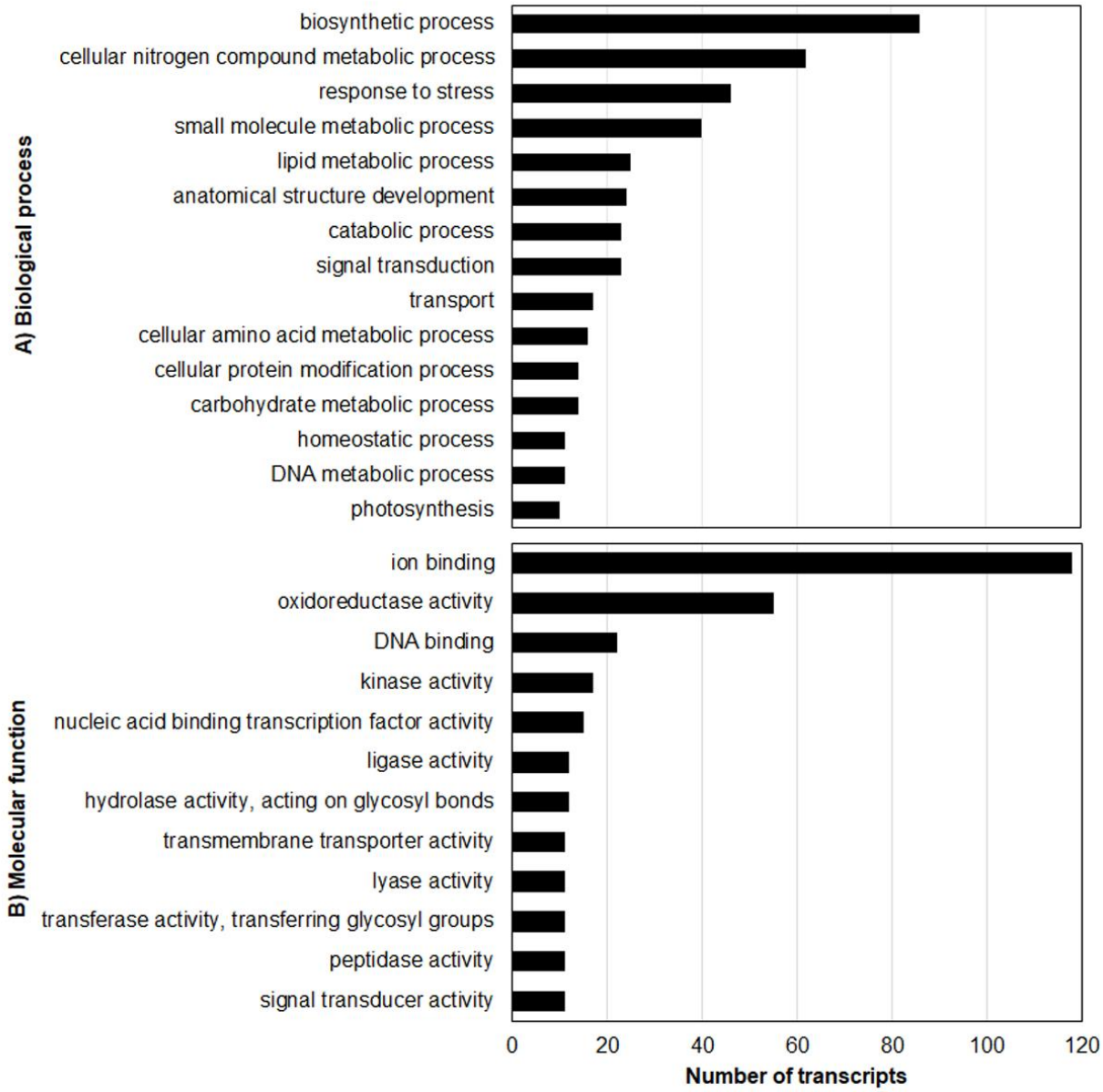

Figure 10. Biological processes (A) and molecular functions (B) of differentially expressed genes that are common in treated T relative to treated S and non-treated T plants.

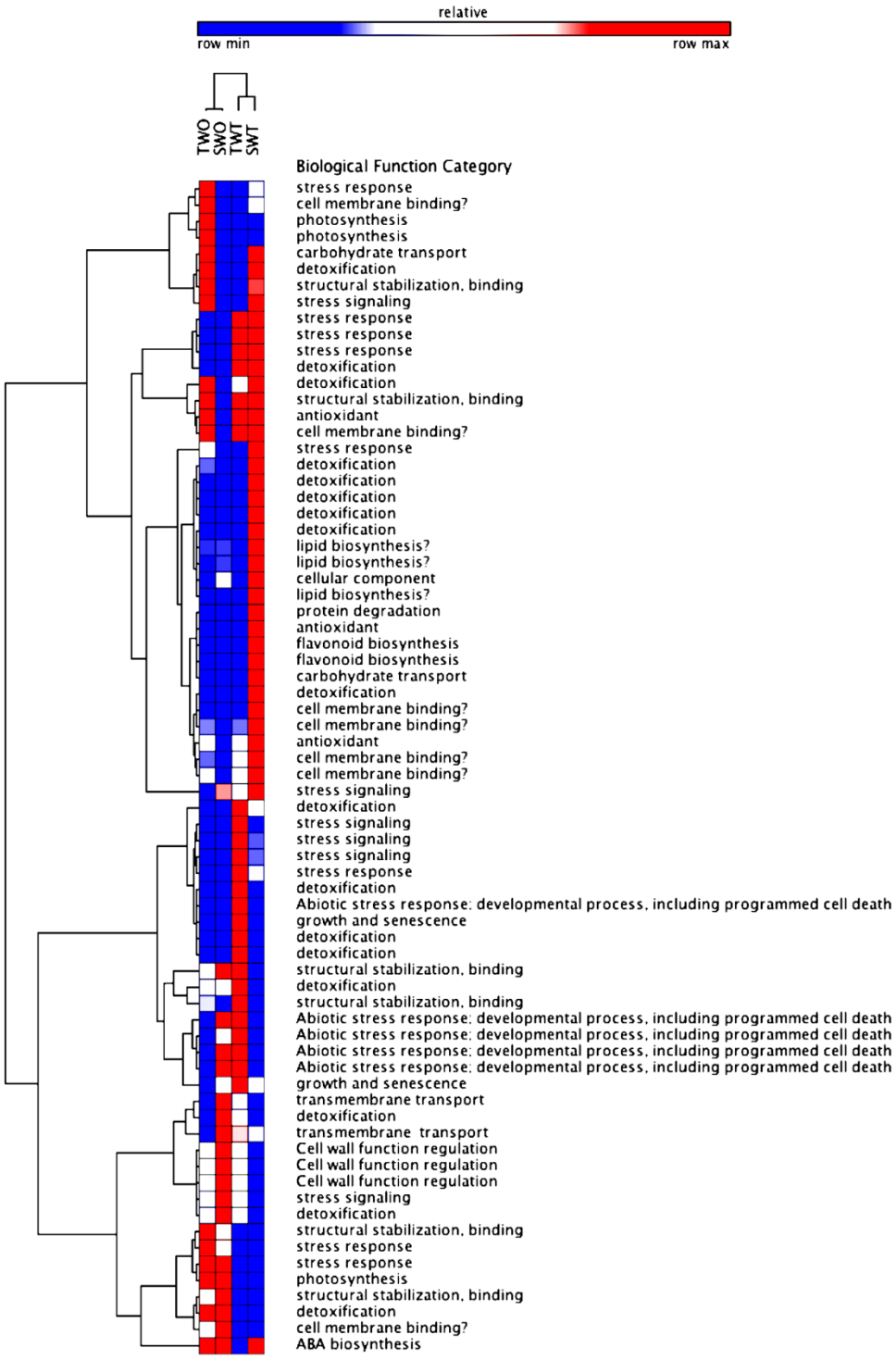

Figure 11. Heat map analysis of genes that are putatively related to abiotic stress response in *A. palmeri*.TWO (non-treated tolerant), SWO (non-treated susceptible), TWT (glufosinate-treated tolerant), SWT (glufosinate-treated susceptible).

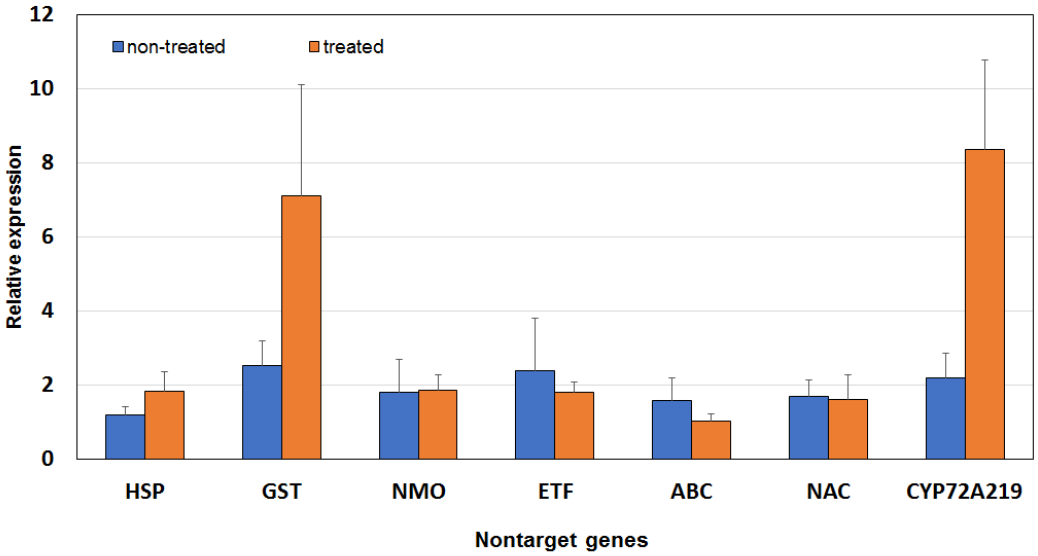

Figure 12. Gene expression fold-change of seven candidate NTS genes in glufosinate-tolerant *Amaranthus palmeri* relative to –susceptible ones. Tolerant and susceptible plants from two populations were identified based on the level of injury incurred after glufosinate treatment. GST and CYP72A219 were highly expressed in the tolerant biotypes in at least one population. Non-treated = before glufosinate treatment, Treated = 24 h after glufosinate treatment. Error bars represent standard error from six biological replicates. HSP = heat shock protein, GST = glutathione S-transferase, NMO = nitronate monooxygenase, ETF = ethylene-responsive transcription factor, ABC = ABC transporter, NAC = NAC transcription factor, CYP72A219 = cytochrome P450 CYP72A219.

CHAPTER IV

MULTIPLE RESISTANCE TO ALS-, PPO, AND EPSPS INHIBITORS IN *AMARANTHUS PALMERI* FROM ARKANSAS, USA

ABSTRACT

Palmer amaranth *(Amaranthus palmeri*) is a major weed problem in agronomic crops in the United States. The majority of the Palmer amaranth populations from Arkansas are resistant to ALS inhibitors. Resistance to ALS inhibitors in Palmer amaranth populations is frequently attributed to target-site mutation, however non-target-site based mechanisms can also be involved. This study aimed to characterize the herbicide resistance profile and investigate target-site and non-target-site resistance mechanisms to ALS inhibitors in a Palmer amaranth population (15Cri-B) from Arkansas. Resistance level (R/S) for flumetsulam, imazethapyr, trifloxysulfuron, fomesafen, and glyphosate were 4.8, 60.9, 4.4, 8.3, and 32 times, respectively, for the 15Cri-B relative to the susceptible population. The addition of cytochrome P450 inhibitor increased trifloxysulfuron phytotoxicity from 25% to 49% with malathion and to 66% with piperonyl butoxide, suggesting the involvement of P450-mediated herbicide metabolism. A $Ser_{653}Asn$ substitution in the *ALS* gene was detected in two out of seven resistant plants tested. Resistant plants without any known resistance-conferring mutations had increased expression of CYP81B and GSTF10 genes after trifloxysulfuron treatment, indicating their involvement in metabolic resistance to ALS inhibitors. This Palmer amaranth population from Arkansas exhibit both target-site and non-target-site based resistance to ALS inhibitors.

INTRODUCTION

Palmer amaranth (*Amaranthus palmeri* S Watson) is a dioecious, aggressive weedy species native to the area of the southwest United States and northern Mexico. It has become one the most damaging and difficult-to-control weeds in major agronomic crops in the United States.[1]Palmer amaranth is considered as an opportunistic, invasive and competitive weed due to its rapid growth rate, continuous emergence period, high fecundity, genetic diversity, and its capacity to evolve traits that increase its potential to grow and reproduce in various cropping systems and environmental conditions.[2,3] With its invasiveness and competitiveness, Palmer amaranth can cause significant yield reduction in cotton (*Gossypium hirsutum*) (77%),[4] soybean (*Glycine max*) (79%)[5], corn (*Zea mays*) (91%),[6] grain sorghum (*Sorghum bicolor*) (63%),[7] and sweet potato (*Ipomoea batatas*) (94%).[8] In addition to reducing yields, its large size (up to 3 m) also decreases harvest efficiency as it hampers the operation of combines harvesters. Palmer amaranth is an obligate outcrossing species which enables it to adapt and spread herbicide resistance genes quickly. To date, various populations had evolved resistance to six different mechanisms of action (MOA), namely: acetolactate synthase (ALS), 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), photosystem II, 4-hydroxyphenylpyruvate dioxygenase (HPPD), microtubule, and protoporphyrinogen oxidase (PPO) inhibitors.[9] Palmer amaranth populations in Arkansas had evolved resistance to ALS inhibitors and glyphosate, and recently to PPO-inhibiting herbicides which limits herbicide options for growers.[10]

Herbicides are a major tool in weed management; however, its usefulness is threatened by the evolution of herbicide-resistant weeds. Herbicide resistance is an evolutionary adaptation in weed species as a consequence of intensive herbicide selection pressure. Herbicide resistance mechanism can be classified as either target-site (TSR) or non-target-site (NTSR). Target-site

mechanisms include structural changes in the herbicide binding-site due to amino acid substitution or increased expression of the target protein.[11] Compared to TSR, NTSR is less often reported and its molecular mechanisms are not well understood. With NTSR mechanism, the number of herbicide molecules reaching the target site is minimized either by reduced herbicide absorption or herbicide translocation or metabolism of the herbicide into non-toxic metabolites. Reduction in herbicide absorption is due difference in cuticle properties or other structural barriers that prevents that affects herbicide retention on the leaves and/or herbicide absorption through the cuticle.[11] Reduced herbicide translocation is characterized by restricted movement of the herbicide within the plant, often due to sequestration of the herbicide into the vacuole or inhibition of cellular transport.[11-14]

Herbicide detoxification usually involves oxidation of the herbicide molecules, usually carried out by cytochrome P450 monooxygenases (P450s), conjugation of the activated xenobiotic using thiols or sugars, transport of the conjugated molecules into the vacuole or extracellular space by active transport, and degradation of the conjugated molecule into the vacuole or extracellular space.[15] NTSR mechanisms involve multiple genes and usually are part of plant stress response.[11] NTSR mechanisms pose a serious concern in weed management because it can confer unpredictable resistance to a broad range of herbicides, including herbicides that are yet to be commercialized.

Acetolactate synthase (ALS) inhibitors have been widely used for Palmer amaranth control since their introduction in 1982.[16] Inhibition of ALS enzyme affects the synthesis of branched-chain amino acids (valine, leucine, and isoleucine), ultimately leading to plant death. There are five chemical families of herbicide that inhibit ALS: sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), sulfonylaminocarbonyltriazolinones (SACTs),

pyrimidinylthiobenzoates (PTBs). The ALS herbicides are widely used because they provide broad-spectrum weed control at low doses, soil residual activity, wide application windows, excellent crop safety and low mammalian toxicity.[17] However, these herbicides have high propensity to select resistant weed populations due to strong selection pressure and high levels of natural variability in the *ALS* gene.[18] The widespread occurrence of Palmer amaranth populations that had evolved resistance to ALS inhibitors has threatened the continued use of ALS-inhibiting herbicides.

Most cases of ALS resistance are due to mutation in the *ALS* gene resulting in an enzyme that is less sensitive to herbicide binding. To date, 29 amino acid substitutions in ALS that confer herbicide resistance were identified at Ala_{122} (5), Ala_{205} (2), Arg_{377} (1), Asp_{376} (1), Gly_{654} (2), Pro_{197} (11), Ser_{653} (3), and Trp_{574}(4) in weed species.[9] Substitution at Trp_{574} confers resistance to IMIs, PTBs, SUs, and TPs,[18] whereas Asp_{376}Glu and Ala_{205}Phe substitutions confer resistance to all five chemical families of ALS inhibitors.[19, 20] Amino acid substitution at Pro_{197} conferred resistance to SUs with low or no cross-resistance to IMIs,[18] whereas substitutions at Ala_{122} or Ser_{653} conferred resistance to IMI herbicides with low-level resistance to SUs.[21, 22] Recent investigation on 20 Palmer amaranth populations from Arkansas revealed TSR mechanism involving Trp_{574}Ser mutation with a few cases of double mutations involving Ala_{122}Thr, Pro_{197}Ala or Ser_{653}Asn.[23]

Another way whereby plants can become resistant to ALS inhibitors is reducing the amount of herbicide reaching ALS to below what is lethal (NTSR). Resistance to ALS inhibitors is rarely attributed to reduced absorption and translocation,[24-26] and in only few cases have they been reported as partial-resistance mechanism.[7, 28] Many studies have attributed enhanced herbicide metabolism to the cytochrome P450 family of proteins by demonstrating that co-

application of the pesticide malathion, a P450 inhibitor, results in loss of resistance or reduced level of resistance.[29-32] An enhanced herbicide metabolism has been reported in ALS-resistant rigid ryegrass (*Lolium rigidum*),[33] wild mustard (*Sinapis arvensis*),[34] and late watergrass (*Echinochloa phyllopogon*).[35, 36] Cytochrome P450 genes, CYP81A6 and CYP76B1, confer resistance to sulfonylurea and phenylurea herbicides, respectively.[37, 38] Multiple resistance mechanisms may also exist in a weed population. Resistance to ALS inhibitor in Spanish corn poppy (*Papaver rhoeas*) and flixweed (*Descurainia Sophia* L.) populations was controlled by both target-site mutation (Pro_{197} substitution) and enhanced herbicide metabolism.[39, 40]

Several studies have identified the involvement of several NTSR genes in conferring herbicide resistance by whole-transcriptome sequencing.[39-42] With the availability of the Palmer amaranth transcriptome,[43] candidate NTSR genes conferring resistance to ALS inhibitors can be validated. The objectives of this study were to determine the resistance profile of a Palmer amaranth population from Arkansas to ALS herbicides, fomesafen, and glyphosate as well as to unravel the resistance mechanism to ALS-inhibiting herbicides.

MATERIALS AND METHODS

Greenhouse experiments were conducted at Fayetteville, Arkansas. One known susceptible (SS) and one ALS-resistant Palmer amaranth population (hereafter referred to as 15Cri-B) were used in the experiments. The SS seeds were collected from a vegetable field in Crawford County, Arkansas and have been used routinely for resistance screening due to its limited exposure to herbicides.[44] The 15Cri-B population was collected from a soybean field in the late summer of 2015. Little is known about the crop management history in this field, except that the field has been planted with continuous soybean for several years. Inflorescence from at

least 10 female plants were collected, dried, threshed, and cleaned for bioassay in the greenhouse.

Cross-resistance to multiple herbicides

Seeds were planted in 28- X 54- cm cellular trays filled with commercial potting soil (Sunshine premix No. 1, Sun Gro Horticulture, Bellevue, WA). The experiment was set up in a randomized complete block design with four replications. Each replication consisted of a tray with 50 seedlings. Treatments included glufosinate at 549 g ha^{-1} (Liberty®, Bayer CropScience LP, Research Triangle Park, NC), fomesafen at 263 g ha^{-1} (Flexstar®, Syngenta Crop Protection LLC, Greensboro, NC) and glyphosate at 840 g ae ha^{-1} (Roundup POWERMAX®, Monsanto Co., St. Louis, MO) and five ALS inhibitors namely; trifloxysulfuron at 8 g ha^{-1} (Envoke®, Syngenta Crop Protection LLC, Greensboro, NC), pyrithiobac at 73 g ha^{-1} (Staple LX®, DuPont Crop Protection, Wilington, DE), flumetsulam at 7 g ha^{-1} (Python®, Dow AgroSciences LLC, Indianapolis, IN), imazethapyr at 70 g ae ha^{-1} (Pursuit®, BASF Corp., Research Triangle Park, NC), and primisulfuron at 40 g ha^{-1} (Beacon®, Syngenta Crop Protection LLC, Greensboro, NC). Pyrithiobac and trifloxysulfuron treatments included 0.25% non-ionic surfactant (NIS) (Induce® non-ionic surfactant, Helena Chemical Co. Collierville, TN) by volume. Imazethapyr was applied with 0.25% (v/v) NIS and 1.4% (w/v) ammonium sulfate (AMS), whereas primisulfuron was applied with 0.25% (v/v) NIS and 2244 g AMS ha^{-1}. Flumetsulam, fomesafen, and glufosinate treatments included 0.5% (v/v) crop oil concentrate (COC) (Agri-Dex® crop oil concentrate, Helena Chemical Co. Collierville, TN), 0.5% NIS and 3366 g AMS ha^{-1}, respectively. Herbicides were applied to 7.5-cm tall seedlings using a compressed air, motorized boom, cabinet sprayer equipped with Teejet XR800067 flat fan nozzle (Teejet spray nozzles;

Spraying Systems Co., Wheaton, IL) calibrated to deliver 187 L ha^{-1} at 269 kPa. Mortality was assessed 21 d after treatment (DAT) and each surviving plant was visually evaluated for injury relative to the non-treated control. Injury was recorded on a scale of 0-100% where 0 had no injury and 100% was dead. Data were analyzed using ANOVA in JMP Pro v.13.

Herbicide dose-response assay

Dose-response experiments were conducted as described by Salas et. al (2016).[44] Seeds were planted in 15-cm-diameter pots filled with commercial potting soil and thinned to 5 plants per pot. The experiment was conducted in a completely randomized design with four replications. Seedlings (7.5-cm tall) were treated with flumetsulam (1X = 7 g ha^{-1}), imazethapyr (1X = 70 g ae ha^{-1}), trifloxysulfuron (1X = 8 g ha^{-1}), glyphosate (1X = 840 g ae ha^{-1}) and fomesafen (1X = 263 g ha^{-1}). The resistant plants were sprayed with eight herbicide doses ranging from a 0X to a 8X dose; susceptible plants were ware sprayed with eight doses ranging from 0X to 2X of the recommended herbicide dose. Visible injury and mortality were evaluated 21 DAT. Plants were cut at the soil surface, dried for 48 h, and weighed. Data were analyzed using JMP Pro v. 13 in conjunction with SigmaPlot v. 13. Nonlinear regression analysis was conducted and the data were fitted with a three-parameter log-logistic model (equation 1) to determine the herbicide dose that would cause 50% control

$$y = c/[1 + e)^{-a\,(x-b)} \quad [1]$$

where *y* is the percent biomass reduction, *a* is the asymptote, *b* is the slope, *c* is the inflection point, and *x* is the herbicide dose.

Whole-plant response to trifloxysulfuron with or without P450 inhibitors

Palmer amaranth plants (15Cri-B and SS) were grown in 15-cm diameter pots filled with commercial soil mixture potting medium. Seedlings were thinned to 5 plants per pot after emergence. Uniform sized-plants (7.5-cm tall) were treated with cytochrome P450 inhibitors malathion (1000 g ha^{-1}) (Hi-Yield Chemical Co., Bonham, TX), carbaryl (1100 g ha^{-1}) (Tessenderlo Kerley Inc., Phoenix, AZ) and piperonyl butoxide (1200 g ai ha^{-1}) (MGK Co., Minneapolis, MN) 45 min before applying trifloxysulfuron at 8 g ha^{-1}. Herbicide and P450 inhibitor applications were made using a laboratory sprayer equipped with a flat fan nozzle (TeeJet spray nozzles, Spraying Systems Co., Wheaton, IL 60189) delivering 187 L ha^{-1}. A non-treated control was also provided for each population. At 21 DAT, plant injury was evaluated visually. Aboveground biomass was harvested and dried at 60ºC for 3 d. Results were expressed as the percent biomass reduction relative to the non-treated control. The experiment was conducted in a completely randomized design in a factorial treatment arrangement with P450 inhibitor and herbicide as the main factors. Each treatment had four replications and the experiment was conducted twice. Data were subjected to analysis of variance in JMP Pro v.13 software. Significant means were separated using Fisher's protected $LSD_{0.05}$.

***ALS* gene sequencing**

Leaf tissues from four sensitive (SS) and seven trifloxysulfuron-resistant (15Cri-B) plants were collected and stored immediately at -80ºC freezer. Genomic DNA was extracted using the modified hexadecytrimethylammonium chloride, previously described by Salas et al.[44] Polymerase chain reaction (PCR) was conducted in a 40 µL mixture of 20 µL 2X PCR master

mix (Takara Bio USA Inc., San Francisco, CA), 2 µL of both forward and reverse primers (10 µM), 2 µL of genomic DNA (50 ng µL-1), and 16 µL water using MJ Research thermal cycler (PTC-200, MJ Research, Inc., Waltham, MA). The following primers were used to sequence the full *ALS* gene (2 kb): forward primer PAALS_F (5'-ATGGCGTCCACTTCAACAAAC-3'), reverse primer PAALS_R (5'-GGTGATGGAAGAAGGGCTTATTAG-3); and internal primers PAALS_F2 (5'-AGGATATTCCTAGAATTGTTAAGG-3'), PAALS_F3 (5'-ATGCGGTTGTAAGTACCGGTGT-3'), PAALS_R1 (5'-CCTGGACCTGTTTTGATTGATA-3'). PCR was performed under the following conditions; initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, then final extension at 72°C for 10 min. The PCR amplification products were resolved on a 0.8% agarose gel to confirm the expected fragment size (2 kb). The PCR product was purified using EZNA gel extraction kit (Omega Bio-Tek, Inc., Norcross, GA) following the manufacturer's instructions. The purified PCR product was sequenced at Eurofins Genomics, Louisville, KY. The resulting DNA sequences were cleaned, aligned and analyzed using Sequencher, BioEdit and ClustalW softwares.

Gene expression using qRT-PCR

The Palmer amaranth populations (15Cri-B and SS) were treated with trifloxysulfuron at 8 g ha^{-1} using the previously described procedure. Leaf tissues were collected 3 days before and 24 h after herbicide treatment. Genomic DNA from leaf tissues were subjected to PCR to amplify the *ALS* gene following the previously described procedure. The purified PCR products were sent for *ALS* gene sequencing. Plant samples which lacked any previously reported *ALS* point mutation were used for the gene expression. The experiment was conducted in a completely randomized

design with herbicide treatment, plant biotype, and gene expression as the variables. Three biological replicates from non-treated and treated samples of 15Cri-B and SS populations were used. Total RNA was extracted from leaf samples using PureLink RNA Mini kit (Life Technologies, Carlsbad, CA). cDNA synthesis was conducted using 5 µg total DNAse-treated RNA using Reverse Transcription System first-strand cDNA synthesis kit (Promega). A 4-fold serial dilution of cDNA samples (1:1, 1:5, 1:25, 1:125) was used to construct a standard curve. Primers were designed to amplify ALS and six NTSR genes that were reported to be associated with resistance to ALS-inhibiting herbicides (Table 1). DEAD-box RNA helicase and β-tubulin were used as internal control for normalization of gene expression [45, 46]

Quantitative real-time PCR (qRT-PCR) was conducted to determine changes in expression of *ALS* and NTSR candidate genes relative to the reference genes. The qRT-PCR experiments were conducted in a 12-µL volume containing 6.25 µL of SyberGreen Master Mix, 1 µL of 1:10 diluted cDNA, and 0.5 µL of 10 µM primers (1:1 mix of forward and reverse primers). Amplification was performed with three technical replicates using a Mastercycler EP Realplex machine (Eppendorf) under the following conditions: 10 min at 94 ºC, 40 cycles of 94 ºC for 15 s and 60 ºC for 1 min, followed by a melt-curve analysis. Data were analyzed using Realplex 2.2 software. Fold-change in gene expression as ($2^{-\Delta Ct}$) was calculated by the comparative method,[47] relative to the susceptible samples, where ΔCT = [C_T target gene –C_t mean of the two internal control genes]. Wilcoxon non-parametric test (α=0.05) was used to determine statistical difference in gene expression between resistant and susceptible biotypes.

RESULTS AND DISCUSSION

Cross-resistance to ALS-, EPSPS- and PPO-inhibitors

The 15Cri-B population was resistant to ALS inhibitors flumetsulam, imazethapyr, primisulfuron, pyrithiobac, trifloxysulfuron with mortality ranging from 0 to 35% (Table 2). Acetolactate synthase inhibitors such as pyrithiobac and trifloxysulfuron were widely used in cotton fields in the past to control broadleaf weed species including Palmer amaranth *s*pecies.[48,49] Similarly, imazethapyr used to be highly effective in managing *Amaranthus* species in soybean fields.[50,51] These once-effective herbicides now fail to provide the desired Palmer amaranth control as observed in numerous fields. Cross-resistance to multiple ALS-inhibiting herbicides is common in Palmer amaranth.[2] Previous studies indicated imidazolinone-resistant populations from Arkansas and Georgia that were also resistant to chlorimuron, diclosulam, and pyrithiobac.[52,53] Herbicide dose-response experiments conducted on whole plants confirmed resistance to ALS inhibitors (flumetsulam, imazethapyr, trifloxysulfuron), fomesafen, and glyphosate. Based on GR_{50}, its resistance level was 4.8-, 60.9-, and 4.4-fold with flumetsulam (triazolopyrimidine), imazethapyr (imidazolinone), and trifloxysulfuron (sulfonylurea), respectively, relative to the SS population. The level of resistance in 15Cri-B is lower than what has been previously reported for other populations of ALS-resistant Palmer amaranth populations.[54,55] A population of Palmer amaranth from Kansas and Argentina had >2,800- and 288-fold resistance to imazethapyr, respectively.[54, 57] Resistant population of Palmer amaranth from Mississippi and Georgia had 8- and 303-fold resistance to pyrithiobac, respectively.[55, 56] The difference in resistance levels is partly due to the sensitivity of the standard susceptible population used in greenhouse bioassay or due to differences in the mechanism of resistance or

both.[58] Even though the15Cri-B population had a lower resistance ratio than other populations, some plants survived at 8X the normal dose of flumetsulam, imazethapyr, and trifloxysulfuron.

The 15Cri-B population exhibited resistance not only to ALS inhibitors but also to fomesafen (PPO inhibitor) and glyphosate (EPSPS inhibitor) herbicides. Fomesafen and glyphosate had 77 and 61% control, respectively, of this population whereas glufosinate killed 91% of the population (Table 2). The resistance level was 8.3-fold with fomesafen and 32-fold with glyphosate, relative to the SS population (Table 3). The mechanism of resistance to fomesafen and glyphosate in 15Cri-B population is target-site based, via amino acid substitution (Arg_{128}Gly mutation in the *PPO* gene) and amplified *EPSPS* copy number, respectively.[10, 59] Populations of Palmer amaranth resistant to both glyphosate and ALS inhibitors are prevalent and have been identified in 10 states, including Arkansas, Arizona, Delaware, Florida, Georgia, Illinois, Maryland, Michigan, Mississippi, Tennessee, and South Carolina.[9,10,55,56] Other case of multiple resistance was also documented such as the Palmer amaranth population from Michigan that was resistant to ALS, EPSPS, and PSII inhibitors.[58] Several Palmer amaranth populations in Arkansas are resistant to glyphosate, ALS-, and PPO-inhibiting herbicides posing serious challenge to growers.[10, 60] With Palmer amaranth resistant to multiple modes of action, limited herbicide options remain for its control. The use of photosystem II and long chain fatty acid inhibitors for pre-emergence and foliar application of glufosinate in glufosinate-resistant crops can be adopted. Herbicide-resistant trait technologies (LibertyLink®, Xtend®, or Enlist®) are available and can be used along with integrated weed management practices such as applying herbicides with different modes of action, crop rotation and other cultural and mechanical practices (e.g. cover crops, tillage, hand weeding).[61]

P450 inhibitor interaction with trifloxysulfuron

The involvement of P450 monooxygenase in metabolic resistance has often been detected when specific P450 inhibitors enhance injury to resistant biotypes when applied in conjunction with the herbicide.[36, 62] The P450 inhibitors carbaryl, malathion, and piperonyl butoxide applied alone caused little to no effect on the growth of Palmer amaranth. Therefore, these compounds, by themselves, are not harmful to this weed. However, plants sprayed with malathion and piperonyl butoxide prior to trifloxysulfuron treatment incurred higher phytoxicity compared to plants treated with trifloxysulfuron alone (Table 4). Biomass reduction was 25% with trifloxysulfuron alone but pretreatment with malathion or piperonyl butoxide increased Palmer amaranth control to 49% and 66%, respectively. On the other hand, pretreatment with carbaryl did not change the plant response compared to trifloxysulfuron alone. The increased efficacy of trifloxysulfuron applied 45 min after malathion or piperonyl butoxide indicates P450-mediated metabolism of the herbicide in resistant plants. Malathion is an excellent synergist for ALS-inhibiting herbicides in ALS-resistant late watergrass, rigid ryegrass, and tall waterhemp (*Amaranthus tuberculatus).*[36, 62, 63] The synergistic interaction of malathion with sulfonylurea herbicide is likely caused by competitive inhibition of cytochrome P450 degradation enzymes.[64] The inhibition of herbicide activity by malathion occurs when atomic sulfur released from the oxygenated organophosphate inhibits the P450 apoprotein.[65] On the other hand, piperonyl butoxide inhibits certain cytochrome P450 enzymes by causing steric hindrance created by its long side-chain in the substrate access channel of the enzyme.[38] Cytochrome P450 enzymes have long been implicated in herbicide metabolism. These enzymes catalyze nonsynthetic modifications, such as oxidation and hydroxylation.[66] In fact, herbicide selectivity in weeds and crops is attributed to the plant's ability to metabolize the herbicide. Herbicide safeners protect

monocot crops from herbicide damage by inducing the production of P450 enzymes, glutathione S-transferase, and glutathione peroxidase which can enhance the herbicide detoxification processes.[65] Hundreds of P450 genes exist in plants genomes, and each P450 participates in various biochemical pathways to produce primary and secondary metabolites.[67] The complexity of cytochrome P450s increases the difficulty of elucidating the genetics of resistance, however, the use of advanced molecular biology and genomics approaches are beginning to provide new information on the identity and role of P450 genes in endowing metabolic resistance in plants.

Sequence analysis of acetolactate synthase gene

Previous researches have shown that amino acid substitution in the *ALS* gene is the most common resistance mechanism in ALS-resistant *Amaranthus* species[18, 23, 68] The *ALS* gene of seven resistant plants from 15Cri-B contained nucleotide changes at multiple loci; however most of the polymorphisms were silent mutations. A guanine to adenine change at position 653 was detected in two resistant plants which resulted in Ser_{653}Asn amino acid mutation (number relative to *Arabidopsis*) (Fig. 1). The other five resistant plants did not have any of the known resistance-conferring mutations to ALS herbicides. Variability in the *ALS* gene within a population was also reported in ALS-resistant Palmer amaranth from Argentina in which five allelic versions of the *ALS* gene were detected.[57] Given the high genetic diversity of Palmer amaranth, it is not surprising to have several genotypes of Palmer amaranth in a population. Our results agree with the findings of other researchers, who also reported that Ser_{653}Asn mutation conferred high-level resistance to imidazolinone herbicides in tall waterhemp and downy brome *(Bromus tectorum*).[69,70] In greenhouse bioassays 15Cri-B had high level of resistance to imazethapyr (60.9-fold), and low level of resistance to trifloxysulfuron (4.4-fold) and

flumetsulam (4.8-fold). The $Ser_{653}Asn$ mutation has been almost exclusively linked to imidazolinone resistance, conferring low resistance to sulfonylurea in some cases,[18] however, 15Cri-B was also cross-resistant to TPs (flumetsulam) and PTBs (pyrithiobac). NTSR mechanisms possibly exist in these plants that would enable broad resistance to ALS herbicides belonging to different chemical families. Furthermore, the presence of plants that carry the $Ser_{653}Asn$ mutation and plants that do not harbor ALS mutation suggest the existence of both TS and NTSR mechanisms in 15Cri-B population.

ALS and NTSR gene expression analysis

Target gene overexpression has been reported to confer resistance to herbicides. Glyphosate-resistant Palmer amaranth populations contain multiple copies of the *EPSPS* gene resulting in overproduction of the target enzyme EPSPS.[71-73] In our experiment, ALS-resistant and –susceptible Palmer amaranth biotypes had similar *ALS* transcript abundance (1-fold) suggesting that the *ALS* gene is not overexpressed in resistant plants. The *ALS* gene expression did not differ before and after trifloxysulfuron treatment in both biotypes. Stable expression of the *ALS* gene has been documented; in fact, it has been used as reference in several gene expression.[72, 74] However, in shortawn foxtail ***(****Alopecuros aequalis)*, the *ALS* copy number is variable.[75] The *ALS* copy number variation in shortawn foxtail is not likely an adaptation for herbicide resistance since the additional *ALS* copies were deemed to be non-functional pseudogenes. In the case of Palmer amaranth, the *ALS* gene was not differentially expressed between ALS-resistant and –susceptible plants, which indicates that other mechanism(s), apart from target gene overexpression, endow resistance to other ALS inhibitors.

The expression patterns of six genes [four cytochrome P450s (CYP72A, CYPA219A, CYP81D, CYP81B), one glycosyltransferase (GT), and one glutathione-S-transferase (GSTF10)] were examined before and after trifloxysulfuron treatment in resistant and susceptible plants. CYP81B and GSTF10 were induced upon trifloxysulfuron application and were not constitutively expressed in non-treated plants (Fig. 2). The expression of GSTF10 and CYP81B in resistant plants ranged from 3- to 5-fold relative to the susceptible plant suggesting their involvement in NTSR to trifloxysulfuron. Cytochrome P450 and glutathione-S-transferase have been implicated in herbicide degradation; the former catalyzes herbicide oxidation and the latter facilitates conjugation with the herbicide metabolite.[11]

The biochemical role of cytochrome P450-mediated herbicide metabolism have been reported in herbicide-resistant rigid ryegrass and late watergrass populations.[42, 62,76,77,79] For example, high expression of CYP81B1, CYP81A21, and CYPA12 were associated with resistance to SUs and TPs in *Lolium* and late watergrass populations.[77,79,80] Several other cytochrome P450 genes have been linked to ALS resistance in various weed populations such as CYP72A254 (also known as CYPA72A219) in late watergrass (bispyribac),[78] CYP81D in black-grass (*Alopecurus myosuroides*) (mesosulfuron and iodosulfuron),[81] CYP94A1, CYP94A2, CYP71A4 and CYP734A6 in shortawn foxtail (mesosulfuron) (Table 5).[41] In addition, many CytP450s conferring resistance to ALS herbicides have been documented in several major crop species, such as CYP81A6 and CYP72A31 in rice (*Oryza sativa*) and CYP81A9 in maize (*Zea mays*) (Table 5).[37,82,83] It was demonstrated that the wheat (*Triticum aestivum*) CYP71C6v1 expressed in yeast (*Saccharomyces cerevisiae*) is able to metabolize several sulfonylurea herbicides such chlorsulfuron and triasulfuron through phenyl ring hydroxylation.[84] The

CYP81B gene identified in Palmer amaranth (15Cri-B) may also confer resistance to multiple herbicides, but this will require further investigation.

Glutathione *S*-transferases (GSTs) are ubiquitous in plants and have defined roles in herbicide detoxification.[85] Differences in glutathione availability and in the portfolio of GST isoenzymes are associated with herbicide selectivity and herbicide resistance.[86] For example, tolerance of maize and giant foxtail (*Setaria faberi*) to atrazine is due to the high levels of GST that facilitate the detoxification of the herbicide via glutathione conjugation.[86, 87] Furthermore, induction of glutathione and GSTs by herbicide 'safeners' is employed commercially to protect crop plants from injury by certain herbicides.[88] GSTs (phi and tau class) were involved *Lolium* resistance to diclofop.[89] Furthermore, GSTA and GST1 were also recently reported to participate in non-target resistance or tolerance to ALS inhibitors in *Lolium* and maize, respectively.[82,90] It was once demonstrated that transgenic tobacco plants (*Nicotiana tabacum*) expressing maize GST1 had enhanced detoxification of the herbicide alachlor.[91] In ACCase-resistant black-grass, GSTF1 catalyzed the conjugation of herbicide to glutathione as well as acted as peroxidase protecting the cell from oxidative damage.[92-94] Transgenic Arabidopsis (*Arabidopsis thaliana*) expressing the black-grass GSTF1 gene (*AmGSTF1*) has improved tolerance to alachlor and atrazine due to increased accumulation of protective flavonoids.[94] Glutathione S-transferases contribute herbicide resistance by detoxifying and transporting herbicide metabolites and/or counteracting oxidative stress. It remains to be investigated whether the GSTF10 of Palmer amaranth is involved in herbicide conjugation or protection from oxidative damage. An ortholog of GSTF10 in *Arabidopsis* (*AtFSTF10*) possesses only transferase activity and not peroxidase activity.[95] Thus, Palmer amaranth GST10 is likely to be involved in detoxification of ALS-inhibiting herbicides by herbicide conjugation with glutathione.

Our study showed ALS target-site mutation ($Ser_{653}Asn$) conferring resistance to trifloxysulfuron in ~29% (2/7) of 15Cri-B Palmer amaranth population. Previous reports associated $Ser_{653}Asn$ mutation with high-level resistance to IMIs and low-level resistance to SUs,[18] however, the 15Cri-B population exhibited resistance not only to IMIs and SUs but to PTBs and TPs. It is likely that resistant plants that carried the *ALS* $Ser_{653}Asn$ mutation may harbor NTSR mechanisms, but this needs to be verified. The majority of the resistant plants did not carry the $Ser_{653}Asn$ mutation but showed induction of NTSR (GSTF10 and CYP81B) genes, which would endow resistance to multiple ALS herbicides. The occurrence of both TSR and NTSR to ALS inhibitors in the same population was also reported in a Palmer amaranth from Kansas.[96] In that study, 30% of the chlorsulfuron-resistant plants harbored *ALS* $Pro_{197}Ser$ mutation and the remaining 70% was deduced to have cytochrome P450-mediated metabolism based on a malathion pretreatment assay.[96] Other weed species displaying both TSR and NTSR to ALS inhibitors include corn poppy,[40] flixweed,[39] and rigid ryegrass populations.[39,40,97] The evolution of TSR and NTSR in weed species predisposes resistance to multiple herbicide families and modes of action. With Palmer amaranth being a prolific and obligate crossing species, plants in a population can accumulate multiple resistance mechanisms which would likely drive resistance evolution faster and is therefore detrimental to chemical weed management.

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Table 1. Primers used in the gene expression analysis by qRT-PCR.[a]

Gene	Primer Sequence	Name	Reference
Cytochrome P450 CYP72A219	GGACAAGAAACTACATCGACA	Cyp72A219_F3	[78]
	TGTTTGCAGGACTTCTTCC	Cyp72A219_R3	
Cytochrome P450 CYP81D	GTTTATACCATTCGGGTCAGG	Cyp81D_F4	[80]
	TTCTCCCATTCTTTCCCATTC	Cyp81D_R4	
Cytochrome P450 CYP81B	AACGACTTCTAGCACAATGG	Cyp81B_F2	[76]
	GTCCTACGTGCTCTAAGATTTC	Cyp81B_R2	
UDP-glycosyltransferase (GT)	GAATTGATGGAGGGAAGGAATG	GT_F3	[39,76]
	CCTTCAACTTTAGTAGCCTCTTG	GT_R3	
Glutathione-S-transferase (GSTF10)	GCTATTTAGCTGGAGACGAC	GST_F1	[76]
	CCCACCATCTACTTACATTCTC	GST_R1	
Cytochrome P450 CYP72A215	TGCACAATCTCTCGAATGG	Cyp72_F2	[76,83]
	GGATCGTGGTGGGTATAAAG	Cyp72_R2	
ALS	GCTGCTGAAGGCTACGCT	ALS_F2	[72]
	GCGGGACTGAGTCAAGAAGTG	ALS_R2	
DEAD-box RNA helicase	TTGGAACTGTCAGAGCAACC	A36_F	[45]
	GAACCCACTTCCACCAAAAC	A36_R	
β-tubulin	ATTCCCTCGGCTTCATTTC	BT_F1	[46]
	TCCCACATTTGCTGAGTTAG	BT_R2	

[a] Herbicide-treated and non-treated Palmer amaranth plants (15Cri-B and SS populations) with three replications per treatment were used for the qPCR assay. The herbicide-treated plants were harvested 24 h after application of trifloxysulfuron at 8 g ha^{-1}.

Table 2. Response of 15Cri-B Palmer amaranth population to the normal dose of ALS (flumetsulam, imazethapyr, primisulfuron, pyrithiobac, trifloxysulfuron), fomesafen, glyphosate and glufosinate herbicides[a].

Mode of Action	**Herbicide**	**Mortality (%)**
Acetolactate synthase (ALS) inhibitor	Flumetsulam	35
	Imazethapyr	21
	Primisulfuron	4
	Pyrithiobac	0
	Trifloxysulfuron	3
Protoporphyrinogen oxidase (PPO) inhibitor	Fomesafen	77
Enolpyruvyl shikimate-3-phosphate synthase (EPSPS) inhibitor	Glyphosate	61
Glutamine synthetase (GS) inhibitor	Glufosinate	91

[a] Flumetsulam (7 g ai ha^{-1}) , imazethapyr (70 g ae ha^{-1}), primisulfuron (40 g ai ha^{-1}), pyrithiobac (73 g ai $ha-^{1}$), trifloxysulfuron (8 g ai $ha-^{1}$), fomesafen (263 g ai $ha-^{1}$), glyphosate (840 g ae ha^{-1}), and glufosinate (549 g ai ha^{-1}) were applied to 7.5-cm tall seedlings. Each herbicide was applied to 200 seedlings.

Table 3. Level of resistance to ALS, PPO, and EPSPS inhibitors in 15Cri-B Palmer amaranth population.

Mode of Action	**Herbicide**	**GR_{50}[a] (g ai ha^{-1})**		**R/S[b]**
		SS[c]	**15Cri-B**	
Acetolactate synthase (ALS) inhibitor	Flumetsulam	1.68 (1.04-2.32)[d]	8.16 (5.24-11.08)	4.8
	Imazethapyr	6.42 (4.45-8.39)	67.35 (48.27-86.43)	60.9
	Trifloxysulfuron	0.85 (0.63-1.07)	3.8 (2.75-4.87)	4.4
Protoporphyrinogen oxidase (PPO) inhibitor	Fomesafen	15.10 (10-21)	125.26 (85-164)	8.3
Enolpyruvyl shikimate-3-phosphate synthase (EPSPS) inhibitor	Glyphosate	11.9 (9.0-14.7)	385 (287-410)	32

[a] Herbicide dose required to cause 50% biomass reduction.
[b] Resistance level (R/S) calculated using the GR_{50} of the resistant population relative to the susceptible population.
[c] SS = susceptible standard population
[d] Values inside the parenthesis denote 95% confidence intervals.

Table 4. Effect of pretreatment with P450 inhibitors[a] on the phytotoxicity of trifloxysulfuron (8 g ha^{-1}) on 15Cri-B Palmer amaranth population.

P450 inhibitor	Biomass reduction relative to the non-treated control (%)	
	No herbicide	With trifloxysulfuron
Carbaryl	0	22
Malathion	5	49
Piperonyl butoxide	0	66
No P450 inhibitor	0	25
$LSD_{0.05}$		
Main effect of P450 inhibitor	10	
Main effect of herbicide	14	
P450 inhibitor x herbicide interaction	16	

[a]P450 inhibitors were applied 45 min before trifloxysulfuron treatment.

Table 5. Cytochrome P450 genes involved in NTSR to ALS inhibitors in various weed and crop species.

Cytochrome P450 gene	Species	Herbicide	Reference
CYP71A	*Alopecurus myosuroides*	iodosulfuron-methyl, mesosulfuron-methyl	[81]
CYP71A4	*Alopecuros aequalis*	mesosulfuron-methyl	[41]
CYP734A6	*Alopecuros aequalis*	mesosulfuron-methyl	[41]
CYP72A254	*Echinochloa phyllopogon*	bispyribac-sodium	[78]
CYP71B	*Alopecurus myosuroides*	iodosulfuron-methyl, mesosulfuron-methyl	[81]
CYP71A31	*Oryza sativa*	bispyribac-sodium	[83]
CYP71C6v1	*Triticum aestivum*	chlorsulfuron, triasulfuron	[84]
CYP81A6	*Oryza sativa*	bensulfuron-methyl	[37]
CYP81A9	*Zea mays*	nicosulfuron	[82]
CYP81A12	*Echinochloa phyllopogon*	bensulfuron-methyl, penoxsulam	[80]
CYP81A21	*Echinochloa phyllopogon*	bensulfuron-methyl, penoxsulam	[80]
CYP81B1	*Lolium*	pyroxsulam, iodosulfuron-methyl, mesosulfuron-methyl	[79]
CYP81D	*Alopecurus myosuroides*	iodosulfuron-methyl, mesosulfuron-methyl	[81]
CYP94A1	*Alopecuros aequalis*	mesosulfuron-methyl	[41]
CYP94A2	*Alopecuros aequalis*	mesosulfuron-methyl	[41]

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SS          Q W L T S G G L G A   M G F G L P A A I G   A A V A R P D A V V   V D I D G D G S F I
15Cri-B_R1  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R2  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R3  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R4  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R5  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R6  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R7  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .

SS          M N V Q E L A T I R   V E N L P V K I M L   L N N Q H L G M V V   Q W E D R F Y K A N
15Cri-B_R1  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R2  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R3  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R4  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R5  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R6  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R7  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .

SS          R A H T Y L G N P S   N S S E I F P D M L   K F A E A C D I P A   A R V T K V S D L R
15Cri-B_R1  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R2  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R3  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R4  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R5  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R6  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .
15Cri-B_R7  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .

SS          A A I Q T M L D T P   G P Y L L D V I V P   H Q E H V L P M I P   S G A
15Cri-B_R1  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . .
15Cri-B_R2  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . .
15Cri-B_R3  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . .
15Cri-B_R4  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . .
15Cri-B_R5  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   . . .
15Cri-B_R6  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   N . .
15Cri-B_R7  . . . . . . . . . .   . . . . . . . . . .   . . . . . . . . . .   N . .
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Figure 1. Amino acid sequence alignment of acetolactate synthase (ALS) in sensitive (SS) and resistant (15Cri-B) Palmer amaranth plant samples. Two resistant plants harbored Ser_{653}Asn (AGC to AAC) mutation.

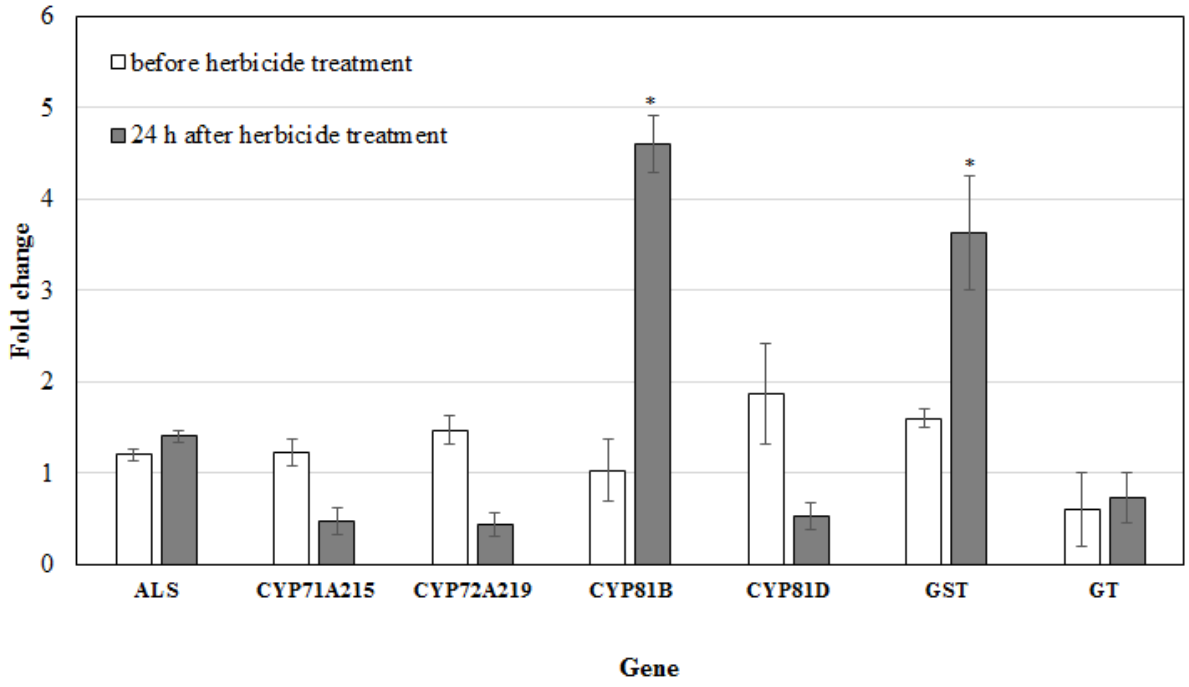

Figure 2. Gene expression analysis of ALS and NTSR genes in trifloxysulfuron-resistant (15Cri-B) and –susceptible (SS) Palmer amaranth biotypes. Fold-change ($2^{-\Delta Ct}$) in gene expression was calculated relative to the susceptible samples, where ΔCt = [Ct target-gene - Ct mean of internal control genes]. Means and standard errors from three biological replicates are shown. Wilcoxon non-parametric test was used to compare differential gene expression between ALS-resistant and susceptible plants. An asterisk denotes fold change significance at $P<0.05$.

CHAPTER V

RESISTANCE TO PPO-INHIBITING HERBICIDE IN PALMER AMARANTH FROM ARKANSAS

Abstract

BACKGROUND: The widespread occurrence of ALS inhibitor- and glyphosate-resistant *Amaranthus palmeri* has led to increasing use of protoporphyrinogen oxidase (PPO)-inhibiting herbicides in cotton and soybean. Studies were conducted to confirm resistance to fomesafen (a PPO inhibitor), determine the resistance frequency, examine the resistance profile to other foliar-applied herbicides, and investigate the resistance mechanism of resistant plants in a population collected in 2011 (AR11-LAW B), and its progenies from two cycles of fomesafen selection (C1 and C2).

RESULTS: The frequency of fomesafen-resistant plants increased from 5% in the original AR11-LAW-B to 17% in the C2 population. The amounts of fomesafen that caused 50% growth reduction were 6-, 13-, and 21-fold greater in AR11-LAW-B, C1, and C2 populations, respectively, than the sensitive ecotype. The AR11-LAW-B population was sensitive to atrazine, dicamba, glufosinate, glyphosate, and mesotrione but resistant to ALS-inhibiting herbicides pyrithiobac and trifloxysulfuron. Fomesafen survivors from C1 and C2 populations tested positive for the PPO glycine 210 deletion previously reported in waterhemp (*Amaranthus tuberculatus*).

CONCLUSION: These studies confirmed that Palmer amaranth in Arkansas has evolved resistance to foliar-applied PPO-inhibiting herbicide.

INTRODUCTION

Palmer amaranth (*Amaranthus palmeri* S. Watson) is one of the most common, troublesome, and economically damaging agronomic weeds throughout the southern United States[1]. This weed continues to emerge throughout the summer making control critical from crop emergence to harvest. The competitive ability of Palmer amaranth is attributed to its fast growth rate[2], high fecundity[3], good light interception, and high water use efficiency[4]. With estimates of over 600,000 seeds per plant, it can replenish the seedbank[3] in one generation. Because it is highly competitive with crops, it can reduce crop yield. Palmer amaranth densities of 8 and 9 plants m^{-2} can reduce soybean yield by 78%[5] and corn grain yield by 91% [6], respectively. Fast et al. (2009)[7] reported that Palmer amaranth interference for 63 d after crop emergence caused 77% cotton yield loss. Apart from reducing yield, more severely in stripper cotton, high infestation of Palmer amaranth interferes with cotton harvest and can increase harvest time by two- to four-fold[8].

Palmer amaranth control has become a challenge because of its high propensity to evolve herbicide resistance, resulting in reduced herbicide options in infested crops such as cotton and soybean. To date, Palmer amaranth has been confirmed resistant to herbicides spanning five modes of action: acetolactate synthase (ALS) inhibitors, carotenoid biosynthesis (4-hydroxyphenylpyruvate dioxygenase) inhibitors, enolpyruvyl shikimate-3-phosphate synthase inhibitor (glyphosate), mitosis inhibitors (dinitroanilines), and photosystem II inhibitors (triazines)[9]. Being dioecious, Palmer amaranth is an obligate cross-pollinated species, allowing herbicide resistance to spread rapidly[10]. Sosnoskie et al. (2012)[11] reported that glyphosate resistance trait was transferred across a distance of at least 300 m through pollen flow.

The widespread occurrence of resistance to ALS inhibitors and glyphosate in Palmer amaranth has led to increasing use of protoporphyrinogen oxidase (PPO)-inhibitor herbicides such as fomesafen, flumioxazin, saflufenacil, and sulfentrazone. Advantageous characteristics of PPO inhibitors include broad herbicidal spectrum as these are active against many monocotyledon and dicotyledon weeds, have low mammalian toxicity, low effective rates, rapid onset of action, and long residual activity on some herbicides in this group[12]. The PPO enzyme catalyzes the conversion of protoporphyrinogen IX (protogen IX) to protoporphyrin IX (proto IX) which is the last common step in the biosynthesis of heme and chlorophyll[13]. In plants, two PPO isoforms are encoded by two different PPO nuclear genes, *PPX1* and *PPX2*. These isoforms share little sequence identity (25%) and differ in their subcellular targeting. PPO1 and PPO2 are localized in plastids and mitochondria, respectively; however, at least some PPO2 isoforms are dual-targeted to both organelles[14-16]. Inhibition of PPO by herbicides results in the generation of singlet oxygen species that attack lipid and protein membranes leading to plant death[17].

The PPO enzyme is inhibited by several herbicide chemical classes (e.g. diphenyl ethers, heterocyclic phenyl ethers, oxadiazoles, phenyl imides, triazolinones and pyrazoles). Herbicides that inhibit PPO have been in the market since the 1960s and were primarily used in soybean. One of these is fomesafen, a diphenyl ether herbicide, which can be applied preplant, preemergence or postemergence for control or suppression of broadleaf weeds, grasses and sedges in soybean. Resistance to PPO herbicides has been slow to evolve (about four decades from first commercialization), and to date, has been confirmed only in seven weed species[9]. The first weed to evolve resistance to PPO herbicides was waterhemp (*Amaranthus tuberculatus*) in 2001[18]. Resistance to PPO herbicides in weedy species has been attributed to target-site mutation in the *PPX2* gene[19-20]. A unique target-site amino acid deletion (Gly_{210}) and Arg_{98}Leu

substitution confers PPO resistance in waterhemp[19] and common ragweed (*Ambrosia artemisiifolia*)[20], respectively.

PPO herbicides are widely utilized for controlling glyphosate-resistant Palmer amaranth in conventional and RoundupReady® soybeans and in cotton. Intensive use of herbicides exerts high selection pressure leading to the evolution of herbicide-resistant weed populations. This paper describes fomesafen-resistance in Palmer amaranth populations from Arkansas.

2 MATERIALS AND METHODS

2.1 Plant materials

In the late summer of 2011, Palmer amaranth samples escaping from either glufosinate or PPO-inhibiting herbicide applications were collected from several fields in Arkansas. Inflorescences from 10 female Palmer amaranth plants per field were collected, dried, threshed, and cleaned for bioassay in the greenhouse. One such field in Lawrence County has been planted with LibertlyLink® soybean and RoundupReady® corn in alternate years since 2011 and treated with either fomesafen and glufosinate or glyphosate. Fomesafen was applied during the soybean production year. Five-hundred mg of seeds from each of the 10 plants per field were mixed to make a composite, which was used for subsequent experiments. Susceptible Palmer amaranth seeds (SS) were collected from a vegetable field in Crawford County, Arkansas. This vegetable field was not exposed to glyphosate or PPO-inhibiting herbicides.

Plants were grown in a greenhouse maintained at 32/25 °C ± 3 °C day/night temperature with a 16-h photoperiod. Plants were watered daily and fertilized with MiracleGro®, a water-soluble all-purpose plant food containing 15-30-15% NPK, every two weeks.

2.2 Fomesafen resistance bioassay in the greenhouse

Palmer amaranth seeds were planted in 28 x 54 cm cellular trays (Redwayfeed Garden and Pet supply, 290 Briceland Rd, Reedway, CA 95560) using Sunshine® premix soil (Sunshine premix #1®, Sun Gro Horticulture, 15831 NE 8th Street, Suite 100, Bellevue, WA 98008). The experiment was conducted in a randomized complete block design with five replications. Each replication consisted of one cellular tray with 50 seedlings, grown at one seedling per cell. The test was repeated. Thus, from each composite seed sample, a total of 500 plants were sprayed with the recommended dose of fomesafen at 264 g ha^{-1} (Flexstar 1.88 EC, Syngenta) when seedlings were 7.5-9 cm tall. The herbicide was applied with 0.5% by volume nonionic surfactant (NIS), using a laboratory sprayer equipped with a flat fan spray nozzle (TeeJet spray nozzles, Spraying Systems Co., Wheaton, IL) delivering 187 L ha^{-1} at 269 kPa. After 21 d, the overall effects of fomesafen (stunting, chlorosis, necrosis, and desiccation) were assessed visually relative to the nontreated control using a scale of 0 to 100 where 0 = no visible injury and 100 = complete death.

Survivors from fomesafen treatment were grown, and allowed to interbreed, to produce C1 and C2 populations of progenies. A subset of nine survivors (which eventually consisted of 3 females and 6 males) from AR11-LAW-B produced the C1 population. C1 plants were sprayed with fomesafen following the same procedure described previously. A subset of six survivors (which eventually consisted of 1 female and 5 males) from C1 plants were grown for seed production to produce C2 population.

2.3 Progression of resistance from the original AR11-LAW-B to C2 Palmer amaranth population

Seeds from the C1 and C2 Palmer amaranth populations were planted in cellular trays to determine the frequency of fomesafen-resistant plants and the progression of resistance from the original population to the C2 population. The experiment was conducted twice in a randomized complete block design with 5 replications; the C1 population was bioassayed four times. In each cellular tray or replication, 50 plants were grown separately and sprayed with 264 g ha^{-1} of fomesafen using the herbicide-application method described previously. Mortality and plant injury were recorded at 21 DAT.

2.3 Fomesafen dose-response bioassay

Palmer amaranth seeds of the susceptible, original AR15-LAW-B, C1, and C2 populations were planted in 11 x 11 cm square pots filled with Sunshine Mix LC1 potting soil (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia, Canada). Seedlings were thinned to five per pot. Seedlings at 7.5-9 cm tall were sprayed with eight doses of fomesafen from 0 to 2109 g ha^{-1}, which corresponds to 0 to 8 times the recommended field dose. The SS population was sprayed with 7 doses from 4 to 264 g ha^{-1} corresponding to 1/64 to 1X recommended dose, with a nontreated check. The herbicide was applied with a 0.5% NIS as described in Section 2.2. The experiment was conducted twice in randomized complete block design with five replications. At 21 DAT, visible injury and the number of survivors were recorded. The above-ground plant tissue was harvested, placed in brown paper bag, dried at 60°C for 48 h, and dry weights recorded. Data were expressed as percentage of biomass reduction relative to the nontreated control. The biomass data generated from two runs were pooled as the test for homogeneity of variance showed that the variance across runs was similar. Regression

analysis was conducted using SigmaPlot v.13. The percent biomass reduction and mortality were fitted to nonlinear, sigmoid, three-parameter Gompertz regression model defined by (Equation 1),

$$y = a*\exp [-\exp \{-b*(x-c)\}] \qquad [1]$$

where Y is the biomass reduction expressed as a percentage of the nontreated control or mortality percentage; a is the asymptote; b is the growth rate; c is the inflection point; and x is the fomesafen dose. The dose needed to kill 50% (LD_{50}) of the population or cause 50% biomass reduction (GR_{50}) was calculated from the above equation.

2.4 Response of Palmer amaranth population to other foliar-applied herbicides

Palmer amaranth seeds from the susceptible and original AR11-LAW-B populations were planted in cellular trays in the greenhouse. Uniform-sized plants (7.5-9 cm tall) were treated with atrazine at 2244 g ha^{-1}, dicamba at 280 g ha^{-1}, glufosinate at 547 g ha^{-1}, glyphosate at 870 g ha^{-1}, mesotrione at 105 g ha^{-1}, and ALS inhibitors. Glufosinate and mesotrione treatments included 3366 g ammonium sulfate (AMS) ha^{-1} and 1% crop oil concentrate (COC), respectively. The ALS inhibitors and their respective rates included pyrithiobac at 73 g ha^{-1} and trifloxysulfuron at 8 g ha^{-1}, applied with 0.25% NIS by volume. Herbicide treatments were applied as described in Section 2.2. Following application, plants were placed on greenhouse benches in a randomized complete block design. Each treatment was replicated twice, with each replication consisting of 50 plants. Mortality was assessed at 21 DAT.

Because AR11-LAW-B was found resistant to pyrithiobac and trifloxysulfuron, dose-response assays were conducted to determine the level of resistance to these herbicides. Seeds were planted in 13-cm round pots filled with commercial potting soil and seedlings were thinned

to five plants per pot. The SS population was sprayed with 8 herbicide doses from 1/16X to 4X the recommended dose of pyrithiobac (1X = 73 g ha^{-1}) and trifloxysulfuron (1X = 8 g ha^{-1}), including a nontreated check. AR11-LAW-B population was treated with eight doses of pyrithiobac (0 to 1166 g ha^{-1}) and eight doses of trifloxysulfuron (0 to 31 g ha^{-1}), which corresponds to 0 to 16X the recommended herbicide dose. Herbicides were applied following the procedure described previously. The experiment was conducted in a completely randomized design with five replications. At 28 DAT, plants were cut at the soil surface, stored in the dryer for 2 d, and the dry weights recorded. Percent biomass reduction relative to the nontreated control were fitted to nonlinear, sigmoid, three-parameter Gompertz regression model. The dose needed to reduce the aboveground biomass by 50% was obtained from the above equation using SigmaPlot v. 13.

2.5 Mechanism of resistance in PPO-resistant Palmer amaranth

Fomesafen survivors from C1 and C2 populations were tested for the presence of the PPO glycine 210 deletion (ΔG_{210}). This deletion confers resistance to PPO herbicides in waterhemp, a relative weedy species of Palmer amaranth[19]. Young leaf tissues from 81 C1 and 13 C2 plants that survived the application of 264 g fomesafen ha^{-1} were collected and stored at -80°C. Tissues from three sensitive plants (SS) were also collected for DNA extraction. Genomic DNA from 100 mg leaf tissue was extracted using hexadecytrimethylammonium bromide (CTAB) method[21] following the modification of Sales et al[22]. The extracted genomic DNA was quantified using a NanoDrop spectrophotometer model ND-1000 (Thermo Scientific, Wilmington DE).

The ΔG_{210} codon deletion was detected using an allele-specific PCR assay as previously described for waterhemp[23]. The same assay was predicted to work for Palmer amaranth based on PPX2 sequence data previously generated from this species[24].

3 RESULTS AND DISCUSSION

3.1 Progression of PPO-resistance in PA-AR11-LAW-B population

The frequency of fomesafen-resistant plants increased from 5% in the original AR11-LAW-B to 17% in the C2 population, in response to the 264 g ha^{-1} dose of fomesafen. Of the 500 plants treated, 25 survived in the original population. The number of survivors increased in the C1 (n=56) and C2 (n=86) population. By the practical description of a resistant population[25], the field population in 2011 (5% resistant individuals) would still be considered a susceptible population. The few remnant plants in the field did not cause any economic loss nor were noticed by the farmer. Until the number of resistant plants reach a level that presents a problem for management and yield loss, the population would not be considered resistant[25]. This current work showed that once a few individuals carrying the resistance trait is selected, sustained herbicide selection pressure on a prolific species like Palmer amaranth could produce a resistant population in two years. This would be when the farmer would call for assistance from crop advisors or Extension Service (personal experience). By the time a population-level resistance is noticed, it is already too late for that field; by then the resistant plants would have had already deposited a large amount of seeds in the soil and the resistant allele(s) would remain in the population.

Not all progenies of fomesafen-resistant survivors carried the PPO-mutation as shown by the resistance frequencies. This suggests that the resistance trait is segregating and progenies are heterogeneous. Palmer amaranth has wide genetic variability owing to its cross-pollinating

behavior. However, for every round of selection, the population becomes more homogeneous. The C2 population had more frequency of resistant plants than C1 or its parent population AR11-LAW-B. After two cycles of selection, the frequency of resistant plants increased about three times. There are examples of rapid evolution of herbicide resistance in response to intense and sustained selection pressure[26]. The Weed Science Society of America (WSSA) defines herbicide resistance as the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide that is normally lethal to the wild type[27]. In herbicide resistance, there is a change in the weed population response (i.e. reduced efficacy of the herbicide) with time as observed with AR11-LAW-B. Thus, the field-collected sample in 2011 was in the early state of resistance evolution to PPO herbicides. The large-scale testing of our Palmer amaranth collection (2008 – 2011) for differential tolerance to fomesafen was conducted in 2013. The tests for heritability and confirmation of G_{210} deletion among fomesafen survivors were completed in 2015. Thus, the detection and confirmation of resistance to PPO herbicides in Palmer amaranth happened years later from collection of the resistance-bearing population.

The farmer of AR11-LAW-B field adopted a corn-soybean crop rotation system. In 2015, the field was clean, except for a sparse remnant Palmer amaranth (about 1 ha^{-1}). Three plants were sampled and tested for resistance to fomesafen; one of these tested positive for the G_{210} deletion mutation. This demonstrates that once the resistant allele has been selected for, it could remain in the population unless the seedbank is depleted.

3.2 Resistance level to fomesafen

Increasing the dose of fomesafen reduced the dry weight of SS, AR11-LAW-B, C1, and C2 populations. The fomesafen dose that caused 50% growth reduction or GR_{50} ($\pm$ 1 standard error) was 13 (+ 0.86), 82 (+ 6.1), 168 (+ 11.9), and 265 (+ 20.4) g fomesafen ha^{-1} for SS, AR11-

LAW-B, C1, and C2 population, respectively (Figure 1, Table 1). Based on these GR_{50} values, the level of resistance to fomesafen in AR11-LAW-B, C1, and C2 population was 6-, 13-, and 21-fold relative to the sensitive population (SS). The GR_{50} increased from 82 g ha^{-1} in the original AR11-LAW-B to 265 g fomesafen ha^{-1} in the C2 population. The commercial field dose of fomesafen (264 g ha^{-1}) is required to reduce aboveground biomass by 50% in the C2 population indicating that the normal field dose would no longer provide effective control. After two more cycles of selection from the point where 5% resistant individuals were detected, the GR_{50} increased three times, reflecting the observed increase in the frequency of resistant plants.

The herbicide dose that caused 50% mortality (LD_{50}) was 16 (+ 1.3), 45 (+ 8.8), 181 (+ 16.8), and 262 (+ 30.1) g fomesafen ha^{-1} for SS, AR11-LAW-B, C1, and C2 population, respectively (Figure 2, Table 1). On the basis of LD_{50} values, AR11-LAW-B, C1, and C2 populations had 3-, 11-, and 16-fold resistance relative to the SS population. The LD_{50} values of C1 and C2 populations were relatively similar to the GR_{50} values. The 0.7X and 1X of the commercial field dose of fomesafen would kill 50% of the C1 and C2 population, respectively. Thus, the normal field dose would not control all plants in the C1 and C2 populations, allowing the resistant plants to proliferate progressively in the next growing season. The PPO-resistant common waterhemp in Kansas had about the same GR_{50} value (270 g fomesafen ha^{-1}) as that of the C2 population[18]. Although AR11-LAW-B had higher GR_{50} and LD_{50} than SS, the commercial dose of fomesafen can still kill >90% of the AR11-LAW-B population. However, continued selection had shifted the population response in just two cycles. If each cycle of selection represents one cropping season, we can predict that resistance to PPO herbicides in the source field (in 2011) would be apparent in 2013. Unaware of the impending resistance problem in the field, the farmer happened to have adopted a RoundupReady corn-LibertyLink soybean

cropping system, and kept the field clean, except for a few remnant Palmer amaranth in 2015 (1/3 of which, still carried the resistance trait). Thus, prevention of seed production of survivors cannot be overstated. With good management practices, the evolution of resistant populations can be slowed down.

3.3 Response of Palmer amaranth population to other foliar-applied herbicides

The AR11-LAW-B population was found to be susceptible to atrazine, dicamba, glufosinate, glyphosate, and mesotrione but resistant to ALS inhibitors pyrithiobac and trifloxysulfuron (Table 2). The commercial field dose of pyrithiobac and trifloxysulfuron controlled AR11-LAW-B 17% and 44%, respectively. The GR_{50} for pyrithiobac was 3.2 (+ 1.2) and 44.5 (+ 6.2) g ha^{-1}, respectively, for SS and AR11-LAW-B (Table 3). Similarly, the GR_{50} for trifloxysulfuron for SS and AR11-LAW-B was 0.9 (+0.7) and 5.1 (+ 1.1) g ha^{-1}, respectively. About 0.6X the recommended dose of pyrithiobac and trifloxysulfuron were needed to reduce the aboveground biomass of AR11-LAW-B by 50%. The AR11-LAW-B population had 14-fold resistance to pyrithiobac and 5-fold resistance to trifloxysulfuron. The occurrence of multiple resistances in AR11-LAW-B is not surprising, considering a widespread resistance to ALS inhibitors among Palmer amaranth in Arkansas[28]. With the prevalence of glyphosate-resistant Palmer amaranth population, it is interesting to note that this population is controlled 84% by glyphosate. The grower of this field had been alternating RoundupReady soybean with rice in the past years (before 2011), then shifted to LibertyLink soybean in 2011, and used other management practices for weed control; thus, keeping the population responsive to glyphosate. This population may or may not be resistant to other PPO herbicides, thus further experiments need to be conducted to verify its response to other foliar and soil-applied PPO herbicides. A recent study indicated that soil-applied PPO-inhibitor herbicides remain efficacious in PPO-

resistant waterhemp populations in Illinois, although the length of residual activity is shortened compared with PPO-susceptible waterhemp[29]. With this population evolving resistance to ALS and PPO inhibitors, AR11-LAW-B can still be controlled with atrazine, dicamba, glufosinate, glyphosate, and mesotrione. Reliance on PPO herbicides should be mitigated to hinder or slow the evolution of PPO resistance in Palmer amaranth. Adequate control of Palmer amaranth can be achieved with tank-mixes of residual herbicides such as *S*-metolachlor + metribuzin applied preemergence followed by pyroxasulfone or *S*-metolachlor applied early postemergence. Glufosinate is still an effective option in LibertyLink soybeans; however, it should be coupled with a preemergence program followed by glufosinate tankmixed with *S*-metolachlor or pyroxasulfone early postemergence[30]. In addition, integration of cultural and mechanical control practices would be helpful in managing PPO-resistant Palmer amaranth populations[31].

3.4 Resistance mechanism in PPO-resistant Palmer amaranth

Results revealed 74 out of 81 C1 plants and all 13 C2 samples tested positive for the ΔG210 mutation, whereas all the sensitive plants tested negative for the said mutation (data not shown). This indicated that these survivors were resistant to fomesafen due to target-site mutation in the *PPO* gene. Thinglum et al.[32] reported that ΔG_{210} mutation confers resistance to PPO herbicides in waterhemp populations in Illinois, Kansas, and Missouri suggesting that the ΔG210 mutation is likely the only known mechanism of resistance to PPO inhibitors in waterhemp. Palmer amaranth and waterhemp belong to the *Amaranthus* genus and may, therefore, have some common morphological, biological, and physiological characteristics and genomic tendencies. In fact, based on previous DNA sequence comparisons, it was predicted that the ΔG_{210} mutation would evolve in Palmer amaranth[24]. The loss of a glycine residue in the PPO

gene alters the architecture of the substrate-binding domain of the PPO enzyme[33]. As a result, the mutated PPO enzyme has reduced affinity for several PPO-inhibiting herbicides.

Some survivors that did not show the PPO mutation may harbor other resistance mechanisms such as non-target site-based resistance mechanisms. Diphenyl ether herbicides are detoxified in soybeans by homoglutathione conjugation[34]. Similarly, tolerance to the diphenyl ether fluorodifen in peas is due to rapid conjugation with glutathione[35]. Alternatively, the lack of detection of the ΔG_{210} mutation in some resistant plants could be due to sequence polymorphisms at the primer binding sites, resulting in false negatives.

3.5 Implications and future research

PPO inhibitors have been used heavily in the past years to combat herbicide-resistant Palmer amaranth. Before this, PPO herbicides were among the most widely used herbicides for soybean. As history has shown, intensive use of any herbicides often results in the selection for genes conferring herbicide resistance in weed populations. This research confirmed the occurrence of the first Palmer amaranth population to have evolved resistance to a PPO-inhibiting herbicide. Recurrent selection with the same herbicide significantly increased the frequency of resistant plants. Because resistance to glyphosate is also rampant, it is expected that populations with multiple resistance to PPO and ALS inhibitors as well as PPO inhibitors and glyphosate will evolve. With Palmer amaranth resistant to these modes of action, limited herbicide options remain for Palmer amaranth control. The evolution of resistance to PPO inhibitors in Palmer amaranth is a recent phenomenon in the southern US; thus, best management practices (including diversification of herbicide modes of action) are vital to manage the spread of resistance, especially in soybean and cotton acres. Since PPO resistance

may still be localized, best management practices should be employed on a broader scale immediately.

Future research will investigate the genetics and mechanism of inheritance to PPO inhibitors in AR11-LAW-B C1 and C2 population, the efficacy of other PPO herbicides (soil or foliar) on PPO-resistant populations, the distribution and population genetics of PPO-resistant Palmer amaranth populations and fitness of multiple-resistant plants.

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Table 1. GR_{50} and LD_{50} values of original, C1, and C2 AR11-LAW-B populations in Arkansas, USA.

Population	GR_{50} (g ha^{-1})	R/S[a]	LD_{50} (g ha^{-1})	R/S[b]
AR11-LAW-B	81.8 (± 5.1)[c]	6	44.8 (± 8.8)[c]	3
C1	167.8 (±11.9)	13	180.8 (± 16.8)	11
C2	265.0 (± 20.4)	21	262.5 (± 30.1)	16
SS[d]	12.9 (± 0.8)		16.3 (± 1.3)	

[a]Resistance levels (R/S) calculated using the GR_{50} of the resistant population relative to the susceptible standard.
[b]Resistance levels (R/S) calculated using the LD_{50} of the resistant population relative to the susceptible standard.
[c]Standard error
[d]Herbicide-susceptible standard population

Table 2. Response of *Amaranthus palmeri* AR11-LAW-B population to foliar-applied herbicides other than protoporphyrinogen oxidase inhibitors, Arkansas, USA.

Herbicide	Mortality (%)[a]	Resistance classification
atrazine	100	susceptible
dicamba	100	susceptible
glufosinate	100	susceptible
glyphosate	84	susceptible[b]
mesotrione	94	susceptible
pyrithiobac	17	resistant
trifloxysulfuron	44	resistant

[a]Uniform-sized plants (7.5-9 cm tall) were sprayed with atrazine at 2244 g ha^{-1}, dicamba at 280 g ha^{-1}, glufosinate at 547 g ha^{-1}, glyphosate at 870 g ha^{-1}, mesotrione at 105 g ha^{-1}, pyrithiobac at 73 g ha^{-1} and trifloxysulfuron at 8 g ha^{-1}. Glufosinate and mesotrione treatments included 3366 g ammonium sulfate (AMS) ha^{-1} and 1% crop oil concentrate (COC), respectively. Pyrithiobac and trifloxysulfuron were applied with 0.25% NIS by volume. Mortality was recorded 21 d after herbicide application.

[b]Plants were not dead at evaluation time, but survivors incurred high injury and did not grow to maturity.

Table 3. GR_{50} values and resistance levels to ALS inhibitors in AR11-LAW-B Palmer amaranth population in Arkansas, USA.

Population	Pyrithiobac		Trifloxysulfuron	
	GR_{50} (g ha^{-1})	R/S[a]	GR_{50} (g ha^{-1})	R/S
AR11-LAW-B	44.5 (±6.2)[b]	14	5.1 (±1.1)	5
SS[c]	3.2 (±1.2)	-	0.9 (±0.7)	-

[a]Resistance levels (R/S) calculated using the GR_{50} of the resistant population relative to the susceptible standard.
[b]Standard error of estimate
[c]Herbicide-susceptible standard population

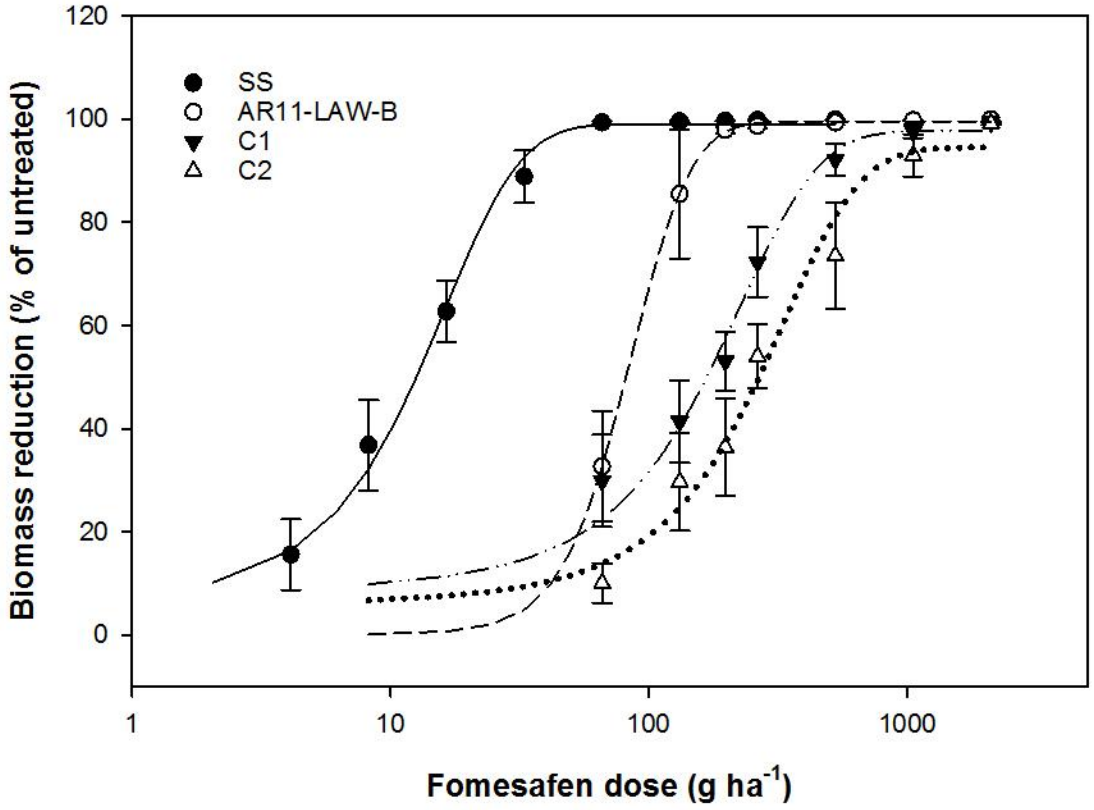

Figure 1. Shoot biomass reduction (%) of PPO-resistant and –susceptible Palmer amaranth population, 21 d after fomesafen treatment.Treatment means (n = 10) + 1 standard error are plotted and fitted to a regression curve. Data were best described with a nonlinear, sigmoidal, three-parameter Gompertz regression function, y =a*exp{-exp[-b*(x-c0]}.

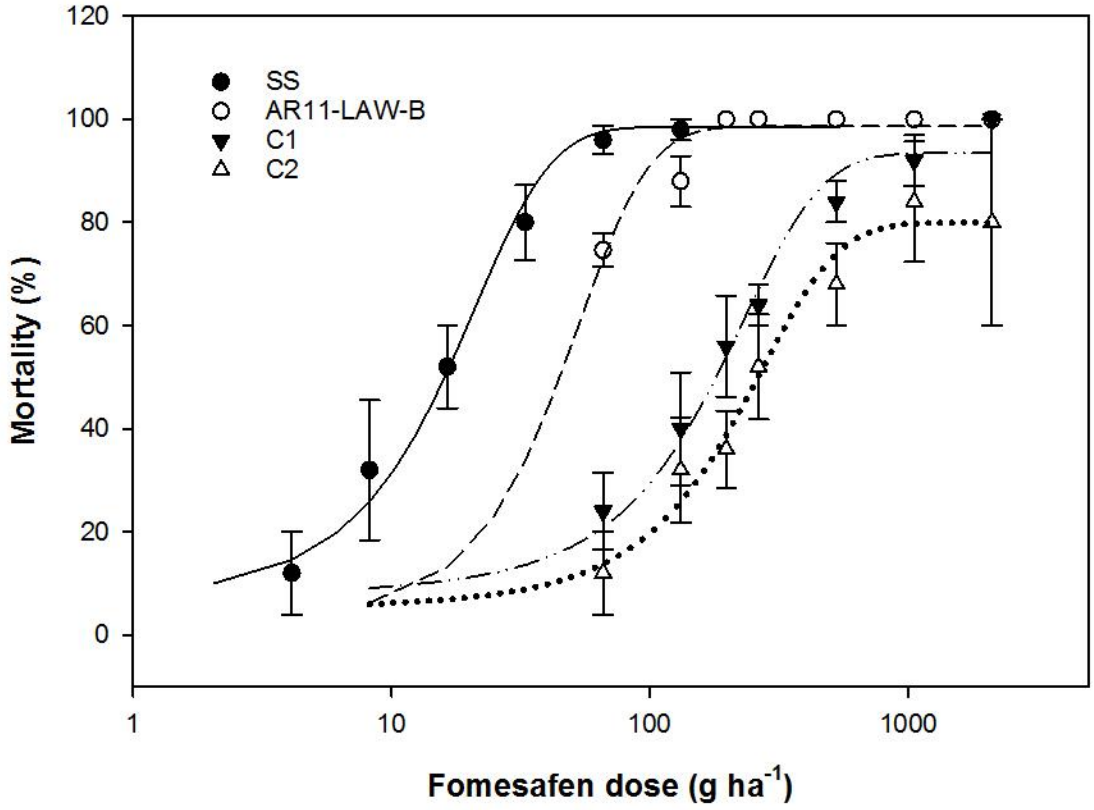

Figure 2. Dose-response curves of PPO-resistant and –susceptible Palmer amaranth population from Arkansas.Mortality (%) were obtained at 21 d after herbicide treatment. Treatment means (n = 10) + 1 standard error are plotted and fitted to a regression curve. Data were best described with a nonlinear, sigmoidal, three-parameter Gompertz regression function, y =a*exp{-exp[-b*(x-c0]}.

CHAPTER VI

FREQUENCY OF GLY-210 DELETION MUTATION AMONG PROTOPORPHYRINOGEN OXIDASE INHIBITOR-RESISTANT PALMER AMARANTH (*AMARANTHUS PALMERI*) POPULATIONS

Abstract

The widespread occurrence of Palmer amaranth resistant to acetolactate synthase inhibitors and/or glyphosate led to the increased use of protoporphyrinogen oxidase (PPO)-inhibiting herbicides. This research aimed to: (1) evaluate the efficacy of foliar-applied fomesafen to Palmer amaranth, (2) evaluate cross-resistance to foliar PPO inhibitors and efficacy of foliar herbicides with different mechanisms of action, (3) survey the occurrence of the PPO Gly-210 deletion mutation among PPO inhibitor– resistant Palmer amaranth, (4) identify other PPO target-site mutations in resistant individuals, and (5) determine the resistance level in resistant accessions with or without the PPO Gly-210 deletion. Seedlings were sprayed with fomesafen (263 g ai ha^{-1}), dicamba (280 g ai ha^{-1}), glyphosate (870 g ai ha^{-1}), glufosinate (549 g ai ha−1), and trifloxysulfuron (7.84 g ai ha^{-1}). Selected fomesafen-resistant accessions were sprayed with other foliar-applied PPO herbicides. Mortality and injury were evaluated 21d after treatment (DAT). The PPX2L gene of resistant and susceptible plants from a selected accession was sequenced. The majority (70%) of samples from putative PPO-resistant populations in 2015 were confirmed resistant to foliar-applied fomesafen. The efficacy of other foliar PPO herbicides on fomesafen-resistant accessions was saflufenacil>acifluorfen = flumioxazin>carfentrazone = lactofen >pyraflufen-ethyl>fomesafen>fluthiacet-methyl. With small seedlings, cross-resistance occurred with all foliar-applied PPO herbicides except saflufenacil (i.e., 25% with acifluorfen, 42% with flumioxazin). Thirty-two PPO-resistant accessions were multiple resistant to glyphosate and trifloxysulfuron. Resistance to PPO herbicides in Palmer amaranth occurred in at least 13 counties in Arkansas. Of 316 fomesafen survivors tested, 55% carried the PPO Gly-210 deletion reported previously in common waterhemp. The PPO gene (PPX2L) in one accession (15CRI-B), which did not encode the Gly-210 deletion, encoded an Arg-128-Gly substitution.

The 50% growth reduction values for fomesafen in accessions with Gly-210 deletion were 8- to 15-fold higher than that of a susceptible population, and 3- to 10-fold higher in accessions without the Gly-210 deletion.

Introduction

Palmer amaranth is one of the most troublesome and economically damaging agronomic weeds in the southern United States. It is able to adapt to diverse climatic and agricultural conditions. The photosynthetic rate of Palmer amaranth (81μmol $m^{-2}s^{-1}$) is three to four times that of corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and soybean [*Glycine max* (L.) Merr.] (Ehleringer 1983). This high photosynthetic rate translates into its rapid growth rate of 5cm day^{-1} under optimum growing conditions (Horak and Loughin 2000). Consequently, this rapid growth rate results in a narrow window of opportunity for effective POST herbicide application. Palmer amaranth control becomes difficult when it is >10-cm tall (Riar et al. 2013). A female Palmer amaranth can produce up to 1 million seeds; however, its seeds are relatively short-lived in the soil, with about 80% mortality in 3yr (Sosnoskie et al. 2014). With its fast growth rate, high fecundity, season-long emergence, high photosynthetic rate, and high propensity to evolve herbicide resistance, Palmer amaranth has become a serious weed in row crops and vegetables (Ehleringer et al.1997; Guo and Al-Khatib 2003; Jha and Norsworthy 2012; Steckel 2007). Palmer amaranth infestation can reduce corn, cotton, and soybean yield 91%, 77%, and 78%, respectively (Bensch et al. 2003; Fast et al. 2009; Massinga et al. 2001). In the past decade, reports abound of Palmer amaranth evolving resistance to glyphosate and other herbicides (Culpepper et al. 2006; Jha et al. 2008; Norsworthy et al. 2008). To date, Palmer amaranth has been confirmed resistant to acetolactate synthase (ALS) inhibitors (Burgos et al. 2001; Horak and Peterson 1995), dinitroanilines (Gossett et al. 1992), 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (Jhala et al. 2014), glyphosate (Culpepper et al. 2006; Norsworthy et al. 2008), photosystem II herbicides (Vencill et al. 2011), and most recently, protoporphyrinogen oxidase (PPO) inhibitors (Salas et al. 2016).

Several chemical families inhibit PPO activity, including diphenylethers (e.g., acifluorfen, fomesafen, lactofen, oxyfluorfen), N-phenylphthalimide (e.g., flumioxazin, flumiclorac), phenylpyrazoles (e.g., fluazolate, pyraflufen-ethyl), oxadiazole (e.g., oxadiazon), oxazolidinones (e.g., pentoxazone), pyrimidinediones (e.g., saflufenacil), thiadiazole (e.g., fluthiacet-methyl), and triazolinone (e.g., carfentrazone, sulfentrazone) (Heap2017). Diphenylether herbicides are used PRE or POST. Some triazolinones and N-phenylphthalimide have high soil activity and are phytotoxic to the crop when applied foliar, hence most are commonly used PRE (Dayan and Duke 2010). There are two known nuclear PPO genes in plants, *PPX1* and *PPX2*, which encode plastid- and mitochondrial-targeted PPO isoforms, respectively; however, some *PPX2* isoforms are dual targeted to both organelles (Lermontova and Grimm 2000; Lermontova et al. 1997; Watanabe et al. 2001). The inhibition of PPO causes accumulation of protophorphyrinogen IX, which leaks from the plastid to the cytoplasm, where it is oxidized rapidly into photosensitive protoporphyrin IX (Becerril and Duke 1989; Jacobs et al. 1991; Lee et al. 1993). Upon exposure to light, protoporphyrin IX generates singlet oxygen molecules that cause lipid peroxidation, membrane destruction, and ultimately cell death (Becerril and Duke 1989; Jacobs et al. 1991). The first weed species to evolve resistance to PPO-inhibiting herbicides was common waterhemp in 2001 (Shoup et al. 2003). Resistance to PPO inhibiting herbicides in Palmer amaranth was first reported in Arkansas and is confirmed also in Tennessee and Illinois (Heap 2017; Salas et al. 2016; L Steckel and K Gage, personal communication). When this research was conducted, resistance to PPO herbicides in *Amaranthus* spp. was attributed to a target-site mutation in the *PPX2* gene only (Patzoldt et al. 2006; Salas et al. 2016). The Gly-210 deletion mutation was present in all PPO-resistant waterhemp populations in Illinois, Kansas, and Missouri (Thinglum et al. 2011). However, a novel PPO

mutation, Arg-98-Leu, was detected in PPO-resistant common ragweed (*Ambrosia artemisiifolia* L.) (Rousonelos et al. 2012).

This study was conducted to investigate the response of Palmer amaranth from Arkansas to various foliar-applied PPO-inhibiting herbicides (acifluorfen, carfentrazone, flumioxazin, fluthiacetmethyl, fomesafen, pyraflufen-ethyl, saflufenacil) and to foliar-applied herbicides with different modes of action (dicamba, glyphosate, glufosinate, trifloxysulfuron). The study also aimed to determine the frequency of Gly-210 deletion among the PPO-resistant biotypes, identify other PPO target-site mutations that endow resistance to PPO-inhibiting herbicides, and compare the resistance level to fomesafen in accessions that contained or lacked the Gly-210 deletion.

Materials and Methods

Plant Materials. A total of 124 Palmer amaranth accessions were collected in the summer between 2008 and 2015 from 23 counties in Arkansas, primarily from the eastern half of the state. Sampling sites were identified by county extension agents and crop consultants. Samples in 2008 and 2009 were collected from 18 counties (17 from eastern Arkansas and 1 from central Arkansas) to survey resistance to glyphosate. Seven counties were sampled in 2011 from fields that were planted with LibertyLink® crops to verify differential response to glufosinate. Fifty-two fields from 16 counties were sampled in 2014. Samples in 2015 (23 accessions) were collected specifically to survey fields with remnant Palmer amaranth after having been sprayed with PPO herbicides. Inflorescences from at least 10 mature female plants−1 field were collected, dried, and threshed. Equal amounts of seeds from each plant were mixed to make a composite seed sample to represent the field. A sensitive standard population (SS) was included in each experiment for comparison. Plants were grown in a greenhouse maintained at 32/25±3°C

day/night temperature with a 16:8-h day:night regime and photon flux density of 0.0005 mol m^{-2} s^{-1}. Plants were planted using commercial potting soil (Sunshine® Premix No. 1; Sun Gro Horticulture, Bellevue, WA), watered daily, and fertilized with Miracle-Gro® (Scotts Miracle-Gro, Marysville, OH) every 2 wk.

Palmer Amaranth Response to Foliar-applied Fomesafen. One hundred-twenty four Palmer amaranth accessions (Table 1) were tested in the greenhouse for resistance to fomesafen. Composite seed samples were planted in 24 by 54 cm cellular trays. Seedlings were thinned to 1 plant $cell^{-1}$ and sprayed with 263g ai ha^{-1} fomesafen (Flexstar®, Syngenta Crop Protection, Greensboro, NC 27419) when seedlings were 7- to 8-cm tall. The herbicide was applied with 0.5% by volume nonionic surfactant (NIS) (Induce®, Helena Chemical, Collierville, TN 38017) using a laboratory sprayer equipped with a flat-fan spray nozzle delivering 187 L ha^{-1} at 221 kPa. The experiment was conducted in a randomized complete block design with two replications and repeated. Each replication consisted of 50 seedlings grown in a cellular tray at 1 seedling $cell^{-1}$. The plants were assessed visually relative to nontreated plants at 21 d after treatment (DAT) using an injury scale of 0 to 100, where 0% = no visible injury and 100% = complete desiccation. Survivors with 0% to 10%, 11% to 30%, 31% to 60%, and 61% to 89% injury were classified as highly resistant, resistant, moderately resistant, and slightly resistant, respectively. Individuals with 90% injury or higher were considered sensitive. In this experiment, an accession with >10% survivors that were at least slightly resistant was considered to be PPO resistant. Data were analyzed using hierarchal clustering in JMP Pro v. 13 (SAS Institute, Cary, NC.).

Response to Other Foliar-applied PPO Herbicides. Twelve accessions that had low mortality (<83%) from foliar-applied fomesafen were bioassayed in the greenhouse for their response to

other foliar-applied PPO herbicides. A sensitive standard accession was also included. Seedlings (100 per accession, 10-cm tall) were treated with the recommended doses of acifluorfen, carfentrazone, flumioxazin, fluthiacet-methyl, lactofen, pyraflufenethyl, or saflufenacil (Table 2). Carfentrazone, pyraflufen-ethyl, flumioxazin, and fluthiacet-methyl treatments included 0.25% NIS (v/v), whereas acifluorfen included 0.125% NIS (v/v). Lactofen was sprayed with 0.5% crop oil concentrate (v/v). Saflufenacil was sprayed with 1% methylated seed oil and 1% ammonium sulfate (w/v). Following herbicide applications, the plants were placed in the greenhouse, grouped by herbicide, and the accessions were randomized within each herbicide group. Mortality and injury of survivors were evaluated at 21 DAT. In the second run of the experiment, herbicides were sprayed when seedlings were 5- to 8-cm tall. Data were analyzed, by herbicide, using JMP Pro v. 13 (SAS Institute, Cary, NC).

Response to Foliar-applied Non-PPO Herbicides. Seventy-three accessions collected in 2014 and 2015 were tested in the greenhouse for response to dicamba, glufosinate, glyphosate, and trifloxysulfuron (Table 2). The SS accession was also included for comparison. The experiment was conducted in a completely randomized design with two replications and two runs. Each replication consisted of 50 plants grown in a cellular tray. Composite seed samples were planted as described earlier. The foliar herbicides were applied to uniform-sized plants (7.6-cm tall). Glufosinate and trifloxysulfuron were sprayed with 3,366g AMS ha^{-1} and 0.25% NIS (v/v), respectively. Herbicide applications were made as described earlier. Plants were evaluated for injury and mortality at 21 DAT. Data were analyzed by herbicide using JMP Pro v. 13 (SAS Institute, Cary, NC). Response to glyphosate was analyzed using hierarchal clustering, collectively considering mortality and injury of survivors.

Mechanism of Resistance to PPO Herbicides. A total of 316 survivors from 47 accessions treated with foliar-applied fomesafen were analyzed for the Gly-210 deletion mutation (Patzoldt et al. 2006; Salas et al. 2016). Young leaf tissues were collected from up to 10 survivors each of 35 accessions sprayed with the recommended dose of foliar-applied fomesafen in the greenhouse bioassays. In addition, leaf tissues were collected from 82 plants in 14 fields with high population density of Palmer amaranth after having been sprayed with PPO inhibitors, among other herbicides. DNA was extracted using a modified Cetyltrimethylammonium bromide (CTAB) protocol (Sales et al. 2008) and quantified using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). An allelespecific PCR assay was used to detect the codon Gly210 following the protocol described for common waterhemp, which also worked for Palmer amaranth (Lee et al. 2008; Salas et al. 2016). Some PPO-resistant plants that did not have the Gly-210 deletion mutation were selected for sequencing of the *PPX2L* gene. Total RNA was extracted using the RNeasy extraction kit (Qiagen 74903, Valencia, CA) and converted to cDNA using the Reverse Transcription kit (Promega A3500, Madison, WI). Initially a partial sequence of the *PPX2L* gene was amplified using the primer pair ppx2-1F (5′-GTAATTCAATCCATTACCCACCTT-3′) and ppx2-3R (5′-TTACGCGGTCTTCTCATCCAT-3′) and sequenced using the internal primers: ppx2-1R (5′-TTCCATACGTCGGGAAATGT-3′), ppx2-2F (5′-TGTTGGAACCATTTCTCTGG-3′), ppx2-2R (5′-GGGGATAAGAACTCCGAAGC-3′) and ppx2-3F (5′-GATGCTGTGGTTGTCACTGC-3′). Eventually, the full-length sequences of PPX2L in PPO-sensitive and PPO-resistant plants were obtained (GenBank accession nos. MF583744 and MF583746) by designing primers based on the upstream and downstream gene sequence of Prince-of-Wales feather (*Amaranthus hypochondriacus* L.) (GenBank accession no. EU024569.1). These additional primers were:

ppx2-5′UTR (5′-TGGCAGATTGAGACAAAATT GG-3′) and ppx2-3 ′UTR (5′-GGCAGAAAAGTCAC TGCACA-3′).

Fomesafen Dose–Response Bioassay. Seven PPO-resistant accessions, including the SS, were used in whole-plant bioassays to determine the resistance level to fomesafen. Three accessions that contained the Gly-210 deletion (14MIS-H, 15MIS-C, 15MIS-E) and four accessions that lacked the Gly-210 deletion mutation (14CRI-C, 15CRI-B, 15PHI-A) were selected. Seedlings were grown in 15-cm-diameter pots and thinned to 5 plants pot^{-1}. Seedlings, 5- to 7-cm tall, were sprayed with 10 doses of fomesafen from 0 to 2,107g ai ha^{-1}. The SS was sprayed with 9 doses from 2 to 263 g ha^{-1}, corresponding to 1/128 to 1X the recommended dose. A nontreated check was included. The herbicide was applied with 0.5% NIS. The experiment was conducted in a randomized complete block design with four replications. At 21 DAT, plants were cut at the soil surface, shoots were dried for 48 h at 60°C, and the dry weights were recorded. Data were analyzed using SAS JMP Pro v.13 (SAS Institute, Cary, NC) in conjunction with SigmaPlot v.13 (Systat Software, San Jose, CA) for regression analysis. The percentage biomass reduction was fit to a nonlinear, sigmoid, four-parameter logistic regression model defined by

$$y = c + [(d - c)/(1 + e\{-a(x - b)\})]$$

where *y* is the biomass reduction expressed as percentage relative to the untreated control, *a* is the growth rate, *b* is the inflection point, *c* is the lower asymptote, *d* is the upper asymptote, and *x* is the fomesafen dose. Estimates of herbicide dose that cause 50% growth reduction (GR_{50}) were determined using the fitted regression equation.

Results and Discussion

Palmer Amaranth Response to Foliar-applied Fomesafen. The frequency distribution of mortality from fomesafen treatment was highly skewed, with a greater proportion of high mortality; therefore, the median values were used to describe the data, except for the 2015 accessions (Figure 1). The 2008 accessions generally incurred 97% mortality with the field dose of fomesafen, except for three accessions with <90% mortality. One rare accession from Phillips County had only 74% mortality; however, the survivors incurred >75% injury and did not produce seeds. Thus, the early samples had individuals that were relatively more tolerant to fomesafen than others. The accessions in 2009 and 2011 were all susceptible, but one of two accessions with 95% mortality, 11LAW-B, contained rare individuals carrying the Gly-210 deletion mutation (Salas et al. 2016). Eleven accessions (22%) collected in 2014 and 16 accessions (70%) in 2015 were classified resistant. The 2015 samples were from fields with suspected resistance to PPO herbicides, and the results confirmed most growers' suspicions. The mortality data with fomesafen treatment was normally distributed from 23% to 100%, with an average of 71%. Accessions classified as PPO resistant in 2015 consisted of individuals with variable response to fomesafen (Figure 2). For example, 15CLA-A had 50% survivors and almost all showed ≤10% injury. On the other hand, 15GRE-A had 75% survivors, and only about one-half showed ≤10% injury. Overall in 2015, 16 accessions from 10 counties showed mostly <90% mortality with fomesafen and were classified resistant based on further evaluations discussed in succeeding sections. This shows that resistance to PPO herbicides among Palmer amaranth has evolved quasi-simultaneously across counties in Arkansas.

Prior to 2015, there were fields with a few Palmer amaranth remaining after PPO herbicide application, but such low numbers did not catch the growers' attention because of the

expected variability in plant response under various environmental conditions. In some cases, any escapes would have been blamed on various factors. Continued selection with different herbicides, but with the same PPO-inhibitor mode of action, has increased the frequency of surviving individuals to a field-scale, observable level. PPO-inhibiting herbicides are used extensively to control glyphosate- and ALS-resistant Palmer amaranth in soybean and cotton (Salas et al. 2016). Prior to the widespread glyphosate-resistance problem in *Amaranthus*, fomesafen had been used primarily as PRE or POST herbicide for broadleaf weed control in soybean. Since fomesafen commercialization in the 1960s, several other herbicides targeting the PPO enzyme have been commercialized. With the expansion of PPO-herbicide use pattern to cotton and PPO-herbicide use in preplant applications, the selection pressure from this mode-of-action group has intensified greatly. Considering that rare PPO-resistant Palmer amaranth individuals were detected retroactively in a population collected in 2011, it took 4 yr before several reports arose of field-level escapes from PPO herbicide application in 2015.

Fields sampled in 2015 were those with remnant infestations of Palmer amaranth after a weed management program that included multiple applications of a PPO herbicide. Most of those fields had plants resistant to 263 g ha^{-1} fomesafen. The frequency of resistance reported here pertained mostly to fields with putative PPO herbicide– resistance problem. A random survey of fields infested with Palmer amaranth across the state, irrespective of cropping history, is expected to produce a lower frequency of resistance to PPO herbicides. Of the 23 fields verified for resistance in 2015, only two had 100% mortality with a field dose of fomesafen. The rest had resistant individuals showing different levels of injury of survivors (Figure 2). The majority of survivors from three accessions (15CLA-A and 15CRI-A) incurred minimal injury

(0% to 10%). High frequency of PPO-resistant individuals among the 2015 accessions threatens the continued use of PPO herbicides.

Multivariate cluster analysis was conducted to group the 2014 and 2015 accessions based on their response to the field use rate of fomesafen, taking into account both mortality data and injury of survivors. The 2014 and 2015 accessions differentiated into four clusters (Figure 3). The 50 accessions belonging to the first cluster were the most sensitive, the majority of which were from the quasi-random collection in 2014. The second cluster is composed of 19 resistant accessions with the majority of survivors showing >60% injury, from the 2014 and 2015 collection. The third cluster comprises two resistant accessions in which most survivors incurred low (11% to 30%) injury. The fourth cluster contains the three resistant accessions in which a large number of survivors incurred the lowest (<11%) injury. This indicates that a large number of PPO-resistant plants incurred low to moderate levels of injury from field use rate of fomesafen, allowing them to mature and reproduce. The evolution of observable, population-level resistance to PPO herbicides in multiple fields in 2015 demonstrated the quasi-simultaneous evolution of resistance to PPO herbicides in Palmer amaranth. This occurred after several decades of use primarily in soybean, then more recently in cotton. The proportion of resistant populations that evolved via gene flow (pollen or seed) or via independent selection is yet to be determined.

Response to Other Foliar-applied PPO-inhibiting Herbicides. The response of Palmer amaranth to other foliar-applied PPO herbicides and to other non-PPO foliar-applied herbicides was evaluated, because PPO herbicides are used heavily to control ALS- and glyphosate-resistant Palmer amaranth. Twelve of the most fomesafen-resistant accessions were tested with other PPO-inhibiting herbicides (Table 3). Mortality with pyraflufen-ethyl and fluthiacet-methyl

was similar across runs; data were therefore combined. These herbicides controlled the SS 100%. The mortality of all accessions, excluding the SS, was <72% with fluthiacet-methyl. Palmer amaranth generally has variable sensitivity to this PPO herbicide and is therefore not listed on the label; whereas this herbicide is expected to provide only partial control of common waterhemp (Anonymous 2011). The mortality rating of all PPOresistant accessions was at least 17% lower than the SS when treated with pyraflufen-ethyl, with the exception of 14CRI-C (>90% mortality). Pyraflufen-ethyl also is naturally weak on Palmer amaranth, although it killed the SS in these experiments, as did the other herbicides.

Mortality was different between runs for acifluorfen, carfentrazone, flumioxazin, lactofen, and saflufencil; data were therefore analyzed separately. In general, mortality was higher in the second run than in the first run due to smaller plant size at the time of herbicide treatment. With acifluorfen, only 2 of 12 fomesafen-resistant accessions tested (14CRI-C and 15CRI-C) were effectively controlled (>90% mortality) in the first run; however, 75% of fomesafen-R accessions were susceptible to acifluorfen when smaller plants were sprayed in the second run. With carfentrazone, all 12 fomesafen-resistant accessions showed only 48% to 83% mortality in the first run. Similar results were observed in the second run. With flumioxazin, eight accessions that had ≥74% mortality in the first run were verified to be sensitive in the second run. About 42% of fomesafen-resistant accessions tested were cross-resistant to foliar-applied flumioxazin. The mortality of all accessions was <70% with lactofen in the first run, with the exception of 15CRI-C (93% mortality). In the second run, all accessions were still poorly to moderately controlled, except for 14CRI-C and 15CRI-C (>90% mortality). Saflufenacil was effective only on five accessions (>90% mortality) with larger seedlings. With smaller seedlings, all 12 fomesafen-resistant accessions showed >97% mortality with saflufenacil. Across all

chemistries, regardless of herbicidal strength, application on small seedlings is necessary for maximum possible efficacy. Application on bigger seedlings is risky. Previous reports indicated that resistance of common waterhemp to foliar-applied PPO herbicides becomes prevalent at the 4- to 6-leaf stage (Falk et al. 2006). Plant size is a critical factor in the efficacy and consistency of performance of most foliar-applied herbicides. When growers miss the application window, selection for resistance is expected to be stronger. If survival in greenhouse bioassays is seen to be consistent across replications and repetitions, then the risk of having survivors in the field populations is high.

Consistent in both runs, fluthiacet-methyl was the least effective and saflufenacil was the most effective of all foliar-applied PPO herbicides. The response to PPO herbicides, in order of decreasing efficacy, was as follows: saflufenacil>acifluorfen = flumioxazin> carfentrazone = lactofen>pyraflufen-ethyl>fluthiacetmethyl. In this series, fomesafen would fall between the last two herbicides. The fomesafen-resistant accessions were generally cross-resistant to other foliar-applied PPO herbicides, with the exception of saflufenacil. However, saflufenacil is not an in-crop option for soybean or cotton. Although saflufenacil showed the greatest efficacy, some accessions already showed reduced sensitivity to the field use rate of saflufenacil when applied to 10-cm-tall seedlings. Previous studies on diphenylether-resistant tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] also showed cross-resistance to other foliar-applied PPO herbicide families (Patzoldt et al. 2005; Shoup et al. 2003; Wuerffel et al. 2015a).

Response to Foliar-applied Non–PPO Inhibiting Herbicides. Palmer amaranth accessions collected between 2014 and 2015 were tested with dicamba, glufosinate, glyphosate, and trifloxysulfuron. The 73 accessions, including the SS accession, differentiated into four clusters in response to glyphosate (Figure 4A). The first cluster was composed of 13 resistant accessions

that had 0% to 36% mortality with glyphosate, with the majority of survivors incurring 31% to 60% injury. Twenty-three accessions constituted the second cluster, with resistance to glyphosate in which a large number of the survivors incurred <11%injury. The third cluster was composed of 24 accessions with ≥65% mortality, 17 of which were classified as sensitive (at least 90% mortality). The fourth cluster included 14 resistant accessions (36% to 68% mortality), in which most of the survivors sustained >60% injury from glyphosate treatment. Overall, about 50% of accessions (Clusters 1 and 2) were resistant to glyphosate, with mortality ranging from 0% to42%. In addition, all accessions were also resistant to trifloxysulfuron, showing <86% mortality (Figure 4B). The widespread occurrence of ALS- and glyphosate-resistant Palmer amaranth in Arkansas was reported previously (Burgos et al. 2009). The ALS-inhibiting herbicides and glyphosate had been used extensively in the past. Overall, the 27 PPO-resistant accessions were also resistant to either ALS inhibitor or glyphosate, or both (Figure 5; Table 4). The majority of PP-Oresistant accessions (85%) exhibited resistance to both glyphosate and trifloxysulfuron.

The 2014 and 2015 accessions were all sensitive to the 549g ai ha^{-1} glufosinate (>90% mortality) (Figure 4B). All accessions incurred >95% mortality, except for 15CRI-B (91%). In the same manner, dicamba controlled all accessions (Figure 4B). Dicamba caused >90% mortality in 61% of the accessions. Mortality of the remaining accessions was <89%; however, the survivors incurred >75% injury. Anecdotal reports, however, indicated poor performance of dicamba in fields infested with PPO-resistant Palmer amaranth.

Prevalence of the *PPO*-Gly-210 Deletion Mutation in Resistant Palmer Amaranth. The Gly-210 deletion in the *PPX2L* gene confers resistance to PPO-inhibiting herbicides in PPO-resistant common waterhemp and Palmer amaranth (Patzoldt et al. 2006; Salas et al. 2016; Wuerffel et al.

2015b). A point mutation in *PPX2* of common ragweed also confers resistance to PPO herbicides; however, whereas a plastid-targeting signal was not found to be encoded by *PPX2* in common ragweed (Rousonelos et al. 2012), *PPX2L* in common waterhemp encodes plastidic and mitochondrial isoforms of PPO (Patzoldt et al. 2006). This dual-targeting phenomenon has been reported previously for spinach (*Spinacia oleracea* L.) and corn (Watanabe et al. 2001). The Gly-210 deletion in imparts herbicide resistance to the dualtargeted protein by altering the architecture of the substrate binding domain (Dayan et al. 2010).

The molecular survey was carried out on 316 fomesafen-resistant plants from 38 accessions representing 13 counties in Arkansas including Clay, Conway, Crittenden, Greene, Independence, Jackson, Lawrence, Lee, Independence, Mississippi, Phillips, White, and Woodruff (Figure 6). The Gly-210 codon deletion mutation was found in 46% and 60% of the survivors from 2014 and 2015 accessions, respectively (unpublished data). The PPO Gly-210 was prevalent among the PPO-resistant accessions; however, a substantial number of the resistant plants did not carry this mutation (Figure 7, A and B). In most of the PPO-resistant accessions (68%), resistant individuals were mixtures of carriers and noncarriers of the Gly-210 deletion. Only 13% of the PPO-resistant accessions (n = 6) contained individuals that were all carriers of the Gly-210 deletion. In nine accessions, none of the resistant individuals carried the Gly-210 deletion. This indicates that either an alternative target-site mutation is present or another resistance mechanism is occurring within and among resistant populations in the field. To verify the occurrence of alternative target-site mutations, we generated a 1,542-bp sequence of *PPX2L* in selected resistant individuals without the Gly-210 deletion (GenBank accession no. MF583745) from the noncarrier accession 15CRI-B. Resistant plants in this accession contained a different amino acid mutation, Arg-128-Gly. This mutation was reported recently in parallel

investigations of PPO-resistant Palmer amaranth, along with an Arg-128-Met mutation (Giacomini et al. 2017). A mutation at the homologous site was identified previously in PPO-resistant common ragweed in the form of an Arg-98-Leu substitution (Rousonelos et al. 2012).

Our survey of more than 300 PPO inhibitor– resistant individuals representing 38 field populations showed that a resistant population may contain a mixture of plants carrying either one of these mutations. Henceforth, testing for these mutations should be done simultaneously on suspected PPO inhibitor–resistant plants using available tools (Giacomini et al. 2017). Sequencing of Palmer amaranth with different resistance levels to PPO inhibitors, with or without the Gly-210 mutation, is ongoing.

The occurrence of other resistance-conferring mutations in other loci of the *PPO* gene is rare, as indicated by previous research. Many single or double point mutations were reported in mutant *PPO* genes in an attempt to obtain PPO herbicide– resistant *Arabidopsis* (Li and Nicholl 2005). Those authors' data showed that single point mutations either provided low resistance or resulted in substantial fitness penalty.

Resistance Level to Fomesafen. The fomesafen dose that caused 50% growth reduction (GR_{50}) ranged from 116 to 232g ha^{-1} in accessions that contained the Gly-210 deletion, whereas GR_{50} ranged from 51 to 153g ha^{-1} in accessions that lacked the Gly-210 deletion (Table 5). Based on these GR_{50} values, the level of resistance to fomesafen relative to the SS ranged from 8- to 15-fold in accessions that contained the Gly-210 and from 3- to 10-fold in accessions that lacked the Gly-210 deletion. The dose–response bioassay indicated high resistance level in accessions carrying the Gly-210 deletion mutation. However, some accessions (14CRI-G and 15CRI-B) that lacked the Gly-210 deletion had GR_{50} values comparable to those of accessions that contained the Gly-210 deletion. Accession 15CRI-B, which contained the Arg-128-Gly mutation, had

similar GR_{50} values to those of the resistant accessions carrying the Gly-210 deletion. This indicates that Gly-210 and Arg-128-Gly mutations result in comparable levels of resistance to fomesafen. Accessions 14CRI-C and 15PHI-A, which lacked the Gly-210 deletion, had lower GR_{50} values than the other PPO-resistant accessions but higher GR_{50} values than the SS. These accessions have not yet been studied for the occurrence of other PPO mutations or other resistance mechanisms. Although our results indicated that the Arg-128-Gly mutation may confer a resistance level similar to that of the Gly-210 deletion, additional data are needed to quantify the impact of this mutation on resistance to PPO herbicides. In addition, we have not ruled out the possibility that other resistance mechanism (s) exist in some PPO inhibitor–resistant accessions, particularly those exhibiting low-level resistance to PPO inhibitors. It is also possible also the populations showing high-level resistance harbor multiple resistance mechanisms, as in the case of ALS-resistant turnipweed [*Rapistrum rugosum* (L.) All.] (Hatami et al. 2016).

The increasing number of Palmer amaranth populations with resistance to PPO herbicides is a great concern, because this limits herbicide options for cotton and soybean. Most PPO-resistant populations were also resistant to glyphosate and trifloxysulfuron, which leaves almost no herbicides for POST Palmer amaranth control. The remaining POST herbicide options for PPO-resistant Palmer amaranth include glufosinate in LibertyLink® crops, dicamba in Roundup Xtend® or Engenia®, or 2,4-D in Enlist Duo® crops. However, overdependence on glufosinate and phenoxy herbicides must be avoided, as some Palmer amaranth populations show high tolerance to dicamba (unpublished data). If we lose glufosinate and dicamba, there will be zero POST options for weed control in soybean and cotton, unless HPPD and 2,4-D traits are commercialized soon.

Overall, the majority of the PPO-resistant individuals carried the PPO Gly-210 deletion. An alternative target-site mutation, Arg-128-Gly, was identified in resistant plants of one accession analyzed that did not carry the Gly-210 mutation. The occurrence of other resistance-conferring mutations or other resistance mechanisms is being investigated. The combination of these mutations in one plant may be lethal, but the presence of these mutations in different plants in one field, or different mutations in proximal fields, may accelerate the evolution and spread of resistance to PPO inhibitors in Palmer amaranth.

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Table 1. Number of Palmer amaranth accessions in Arkansas, USA tested with foliar-applied fomesafen at 263 g ha^{-1}.

Year collected[a]	Number of accessions
2008	25
2009	10
2011	16
2014	50
2015	23
Total	124

[a] Palmer amaranth samples were collected from fields with history of glyphosate, glufosinate, or PPO-inhibiting herbicide use. Sampling in 2015 was done specifically to survey fields with remnant Palmer amaranth population after having been sprayed with PPO herbicides.

Table 2. Common, trade names, and manufacturers of herbicides used in the study.

Site of action	Application	Common name	Trade	Rate	Company	Company website
				g ai ha^{-1}		
PPO	foliar	acifluorfen	Ultra Blazer 2SL	560	United Phosphorus, Inc., King of Prussia, PA 19406	www.upi-usa.com
PPO	foliar	carfentrazone	Aim 2EC	280	FMC Corporation, Philadelphia, PA 19103	www.fmc.com
PPO	foliar	flumioxazin	Valor SX 51 WDG	70.6	Valent USA Corporation, Walnut Creek, CA	www.valent.com
PPO	foliar	fluthiacet-methyl	Cadet 0.91EC	0.672	FMC Corporation, Philadelphia, PA 19103	www.fmc.com
PPO	foliar	lactofen	Cobra 2EC	224	Valent USA Corporation, Walnut Creek, CA	www.valent.com
PPO	foliar	pyraflufen-ethyl	ET 0.2EC	3.64	Nichino America, Wilmington, DE 19808	www.nichino.net
PPO	foliar	saflufenacil	Sharpen 4F	24.6	FMC Corporation, Philadelphia, PA 19103	www.fmc.com
PPO	soil	fomesafen	Reflex 2SL	280	Syngenta Crop Protection, LLC, Greensboro, NC 27419	www.syngenta-us.com
PPO	soil	flumioxazin	Valor SX 51 WDG	70.6	Valent USA Corporation, Walnut Creek, CA	www.valent.com
PPO	soil	saflufenacil	Sharpen 4F	49.3	FMC Corporation, Philadelphia, PA 19103	www.fmc.com
PPO	soil	sulfentrazone	Spartan 4F	280	FMC Corporation, Philadelphia, PA 19103	www.fmc.com

Table 2 (Cont.)

Site of action	Application	Common name	Trade	Rate	Company	Company website
				g ai ha^{-1}		
PPO	soil	oxyfluorfen	GoalTender 4E	280	Dow AgroSciences LLC, Indianapolis, IN 46268	www.dowagro.com
Auxin	foliar	dicamba	Clarity 4SL	280a	BASF Corporation, Research Triangle Park, NC 27709	https://www.basf.com
Glutamine synthetase	foliar	glufosinate	Liberty 280SL	549	Bayer CropScience LP, Research Triangle Park, NC 27709	www.bayer.com
EPSPS	foliar	glyphosate	Roundup PowerMAX 4.5SL	870a	Monsanto Company, St. Louis, MO 63167	www.monsanto.com
ALS	foliar	trifloxysulfuron	Envoke 75	7.84	Syngenta Crop Protection, LLC, Greensboro, NC 27419	www.syngenta-us.com

[a] g ae ha^{-1}

Table 3. Response of fomesafen-resistant Palmer amaranth accessions to the recommended rate of various foliar-applied PPO herbicides.

Accession[a]	Mortality[b]						
	Acifluorfen	Carfentrazone	Flumioxazin	Fluthiacet	Lactofen	Pyraflufen	Saflufenacil
	----------%----------						
14CRI-C	100[c] (100)[d]	84 (99)	100 (100)	71[f]	93 (95)	94[f]	98 (100)
14MIS-H	55 (97)	67 (81)	66 (75)	18	42 (73)	37	79 (100)
15CLA-A	38 (64)	48 (45)	55 (44)	13	23 (66)	29	50 (98)
15CRI-A	68 (94)	54 (55)	74 (90)	39	49 (72)	53	73 (100)
15CRI-C	91 (100)	74 (87)	100 (100)	48	67 (92)	73	92 (100)
15CRI-D	72 (98)	60 (66)	91 (98)	19	44 (75)	60	93 (100)
15GRE-A	31 (78)	66 (72)	48 (81)	21	21 (29)	35	38 (99)
15IND-A	70 (94)	63 (74)	87 (90)	44	70 (79)	64	78 (100)
15LAW-C	73 (93)	58 (84)	81 (98)	33	49 (80)	57	91 (99)
15MIS-D	83 (99)	63 (80)	80 (60)	31	45 (77)	34	89 (100)
15MIS-E	73 (86)	69 (66)	79 (91)	30	54 (78)	54	95 (100)
15MIS-F	60 (93)	67 (67)	68 (89)	21	39 (69)	43	84 (100)

Table 3 (Cont.)

Accession[a]	Mortality[b]						
	Acifluorfen	Carfentrazone	Flumioxazin	Fluthiacet	Lactofen	Pyraflufen	Saflufenacil
	----------%----------						
SS[f]	100 (100)	100 (100)	100 (100)	100	100 (100)	100	100 (100)
$LSD_{0.05}$[g]	14 (15)	19 (19)	15 (19)	23	16 (23)	22	12

[a] Accessions were collected between 2014 and 2015 in Arkansas, USA. Accessions were confirmed resistant to foliar-applied fomesafen (263 g ha^{-1}) except for the susceptible standard (SS).

[b] Mortality ratings from acifluorfen, carfentrazone, flumioxazin, lactofen, and saflufenacil treatments were different across runs, thus data were analyzed separately. Carfentrazone (280 g ha^{-1}), pyraflufen-ethyl (3.64 g ha^{-1}), and flumioxazin (70.6 g ha^{-1}) treatments included 0.25% v/v non-ionic surfactant (NIS). Acifluorfen at 560 g ha^{-1} included 0.125% v/v NIS. Lactofen (224 g ha^{-1}) was sprayed with 0.5% v/v crop oil concentrate. Saflufenacil (24.6 g ha^{-1}) was applied with 1% methylated seed oil and 1% ammonium sulfate.

[c] Values obtained from the first run of experiment. Herbicides were applied to 10-cm tall seedlings.

[d] Values obtained from the second run of experiment. Herbicides were applied to 5- to 8-cm tall seedlings.

[e] Averaged mortality across two runs. Mortality ratings across runs were similar in pyraflufen-ethyl and fluthiacet-methyl treatments, thus data were combined.

[f] SS = sensitive standard population

[g] Fisher's protected LSD to compare accessions within each herbicide

Table 4. Herbicide resistance profile of PPO-resistant Palmer amaranth accessions to other foliar-applied non-PPO herbicides.

Accession	Mortality[a]				
	Fomesafen	Dicamba	Glyphosate	Glufosinate	Trifloxysulfuron
	----------%----------				
14CLA-D	62	97	91	100	85
14CRI-C	82	86	6	100	1
14CRI-G	77	93	85	100	2
14JAC-B	88	97	100	100	61
14LEE-G	86	85	95	100	49
14LEE-J	85	100	0	100	46
14MIS-A	85	97	24	100	44
14MIS-E	69	91	52	100	64
14MIS-G	85	100	89	100	38
14MIS-H	47	98	0	100	67
14PHI-B	83	100	52	100	42
15CLA-A	62	86	14	100	17
15CRI-A	40	87	5	95	7
15CRI-B	77	87	61	91	3
15CRI-C	55	93	5	100	6
15CRI-D	44	76	11	100	4
15GRE-A	23	79	93	99	10
15IND-A	43	83	14	100	22
15LAW-B	88	85	46	96	2
15LAW-C	46	84	13	97	13
15MIS-B	80	87	14	100	1
15MIS-C	64	88	9	99	5
15MIS-D	58	81	14	100	1
15MIS-E	65	84	14	100	8
15MIS-F	56	87	7	100	5
15PHI-A	74	83	2	100	11
15PRA-A	79	79	18	99	11

[a]Fomesafen (263 g ai ha^{-1}), dicamba (280 g ae ha^{-1}), glufosinate (549 g ai ha^{-1}), glyphosate (870 g ae ha^{-1}), and trifloxysulfuron (7.84 g ha^{-1}) were applied to 7.6-cm tall seedlings.

Table 5. GR_{50} values of PPO-resistant Palmer amaranth accessions in Arkansas.

Gly_{210} mutation carrier	Accession	GR_{50}[a]	R/S
		g ai ha^{-1}	
Yes	14MIS-H	232	15
		(148-315)[b]	
Yes	15MIS-C	116	8
		(80-153)	
Yes	15MIS-E	141	9
		(94-188)	
No	14CRI-C	70	4
		(43-97)	
No	14CRI-G	153	10
		(112-195)	
No	15CRI-B	125	8
		(85-164)	
No	15PHI-A	51	3
		(32-70)	
No	SS	15	1
		(10-21)	

[a]GR_{50}: dose of herbicide required to cause 50% biomass reduction
[b]Values in parenthesis indicate 95% confidence intervals.

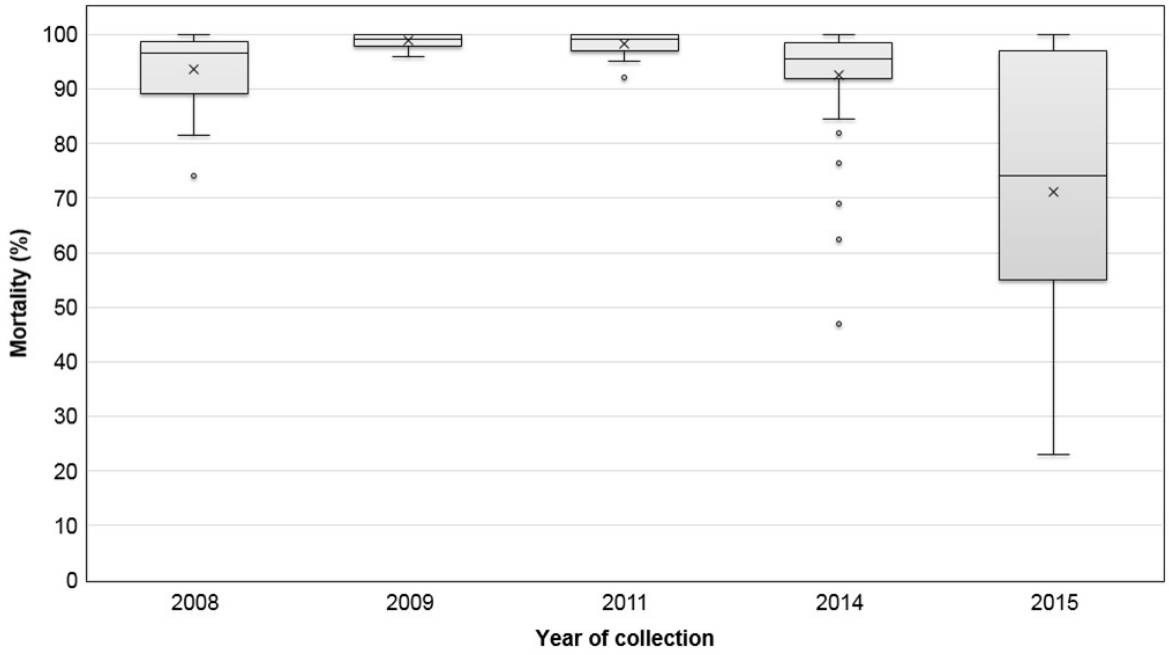

Figure 1. Variability in response to foliar-applied fomesafen (263 g ha^{-1}) among Palmer amaranth accessions collected between 2008 and 2015.Box plot shows median values (horizontal line inside the box), mean values (marked X), first and third quartile values (box-outlines), minimum and maximum values (whiskers), and outlier values (open circles).

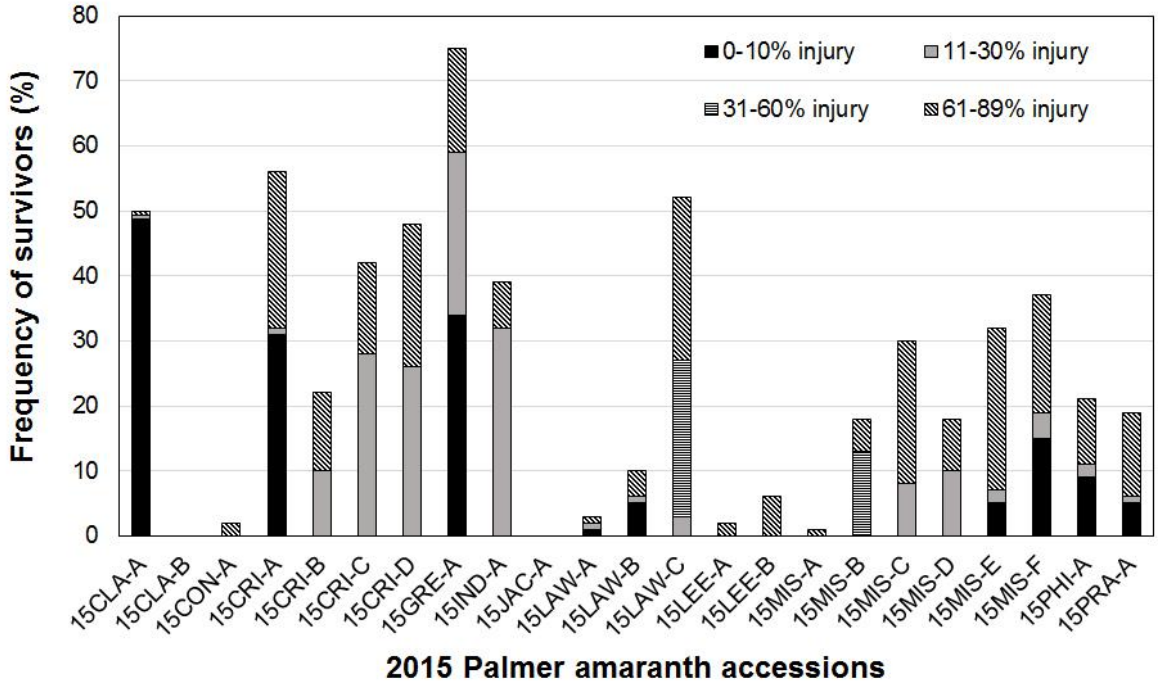

Figure 2. Frequency of fomesafen-resistant plants in Palmer amaranth accessions collected in 2015 from Arkansas.Fomesafen at 263 g ha^{-1} was applied with 0.5% non-ionic surfactant to 7.6-cm tall seedlings. Survivors are categorized based on visible injury.

Cluster	No. of accessions	Mortality (%)			Mean frequency of plants per accession, at different levels of injury (%)				
		Mean	Min	Max	0-10% injury	11-30% injury	31-60% injury	61-89% injury	91-100% injury
1	50	96	88	100	0	0	1	3	97
2	19	68	43	87	2	0	11	15	73
3	2	63	46	80	0	19	2	15	65
4	3	42	23	62	38	0	9	14	40

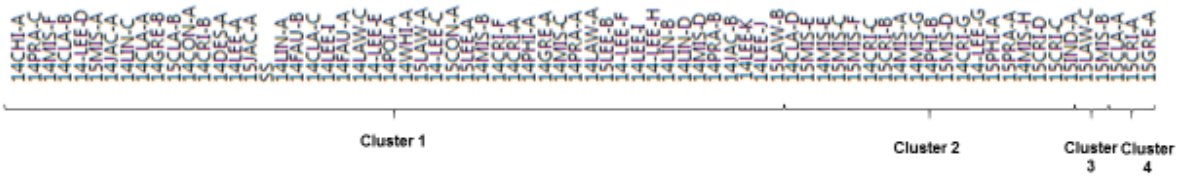

Figure 3. Hierarchal clustering of 2014 and 2015 Palmer amaranth accessions treated with foliar-applied fomesafen at 263 g ha^{-1}.The herbicide was applied with 0.5% non-ionic surfactant to 7.6-cm tall seedlings. Cluster 1 = most sensitive to fomesafen (50 accessions), Cluster 2 = resistant to fomesafen with majority of survivors incurred >60% injury (19 accessions), Cluster 3 = resistant to fomesafen, majority of survivors sustained 11-30% injury (2 accessions), Cluster 4 = resistant to fomesafen, majority of survivors incurred 0-10% injury (3 accessions).

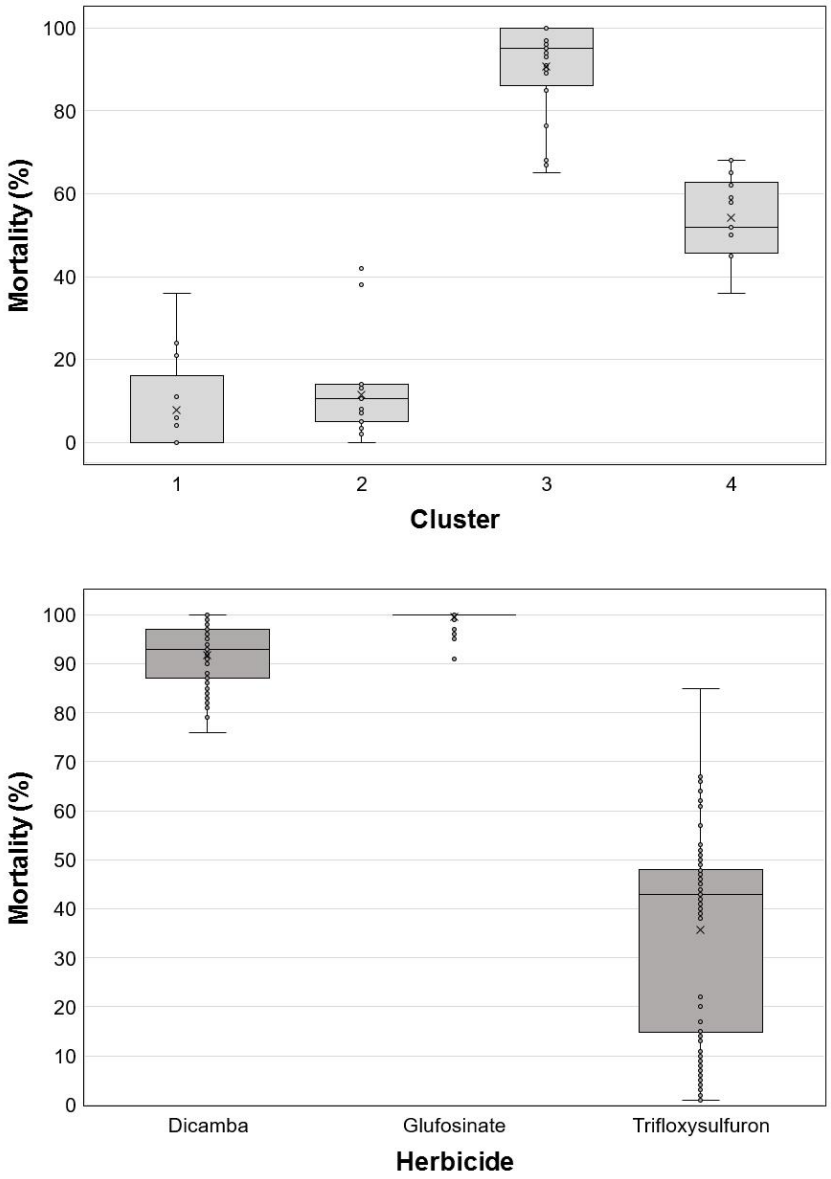

Figure 4. (A) Cluster analysis of mortality levels in Palmer amaranth accessions collected in 2014 and 2015 treated with 870 g ha^{-1} glyphosate. Cluster 1 (n = 13 accessions; resistant to glyphosate with majority of survivors incurred 31-60% injury), cluster 2 (n = 23 accessions; resistant to glyphosate with majority of survivors incurred <11% injury), cluster 3 (n = 23 accessions; least sensitive to glyphosate), and cluster 4 (n = 14 accessions; resistant to glyphosate with majority of its survivors incurred 61-89% injury) are depicted in box-plot. Glyphosate was applied to 7.6-cm tall seedlings. (B) Variability in response to dicamba (280 g ae ha^{-1}), glufosinate (549 g ha^{-1}) and trifloxysulfuron (7.84 g ha^{-1}) among Palmer amaranth accessions collected in 2014 and 2015 from Arkansas, USA. Box plot shows median values (horizontal line inside the box), mean values (marked with X), first and third quartile values (box-outlines), minimum and maximum values (whiskers), and outlier values (open circles).

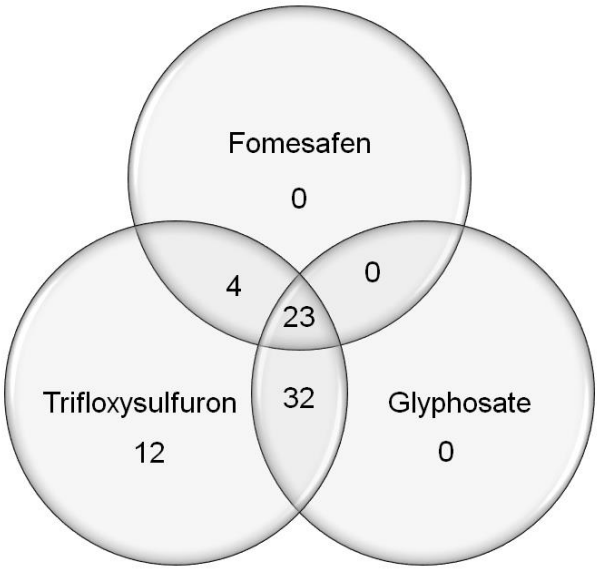

Figure 5. Herbicide resistance profiles of Palmer amaranth populations from Arkansas sampled in 2014 and 2015.

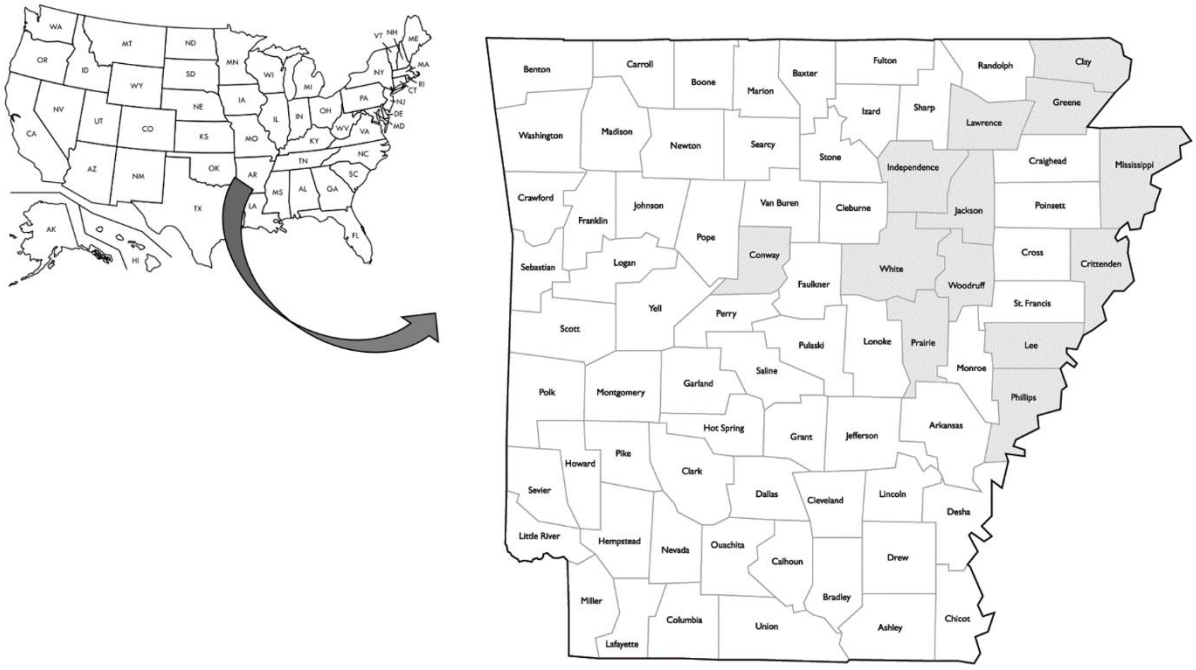

Figure 13. Counties with confirmed PPO-resistant Palmer amaranth in Arkansas.Counties that are shaded had at least one field-population with PPO-resistant Palmer amaranth biotypes carrying the Gly_{210} deletion in the PPO gene.

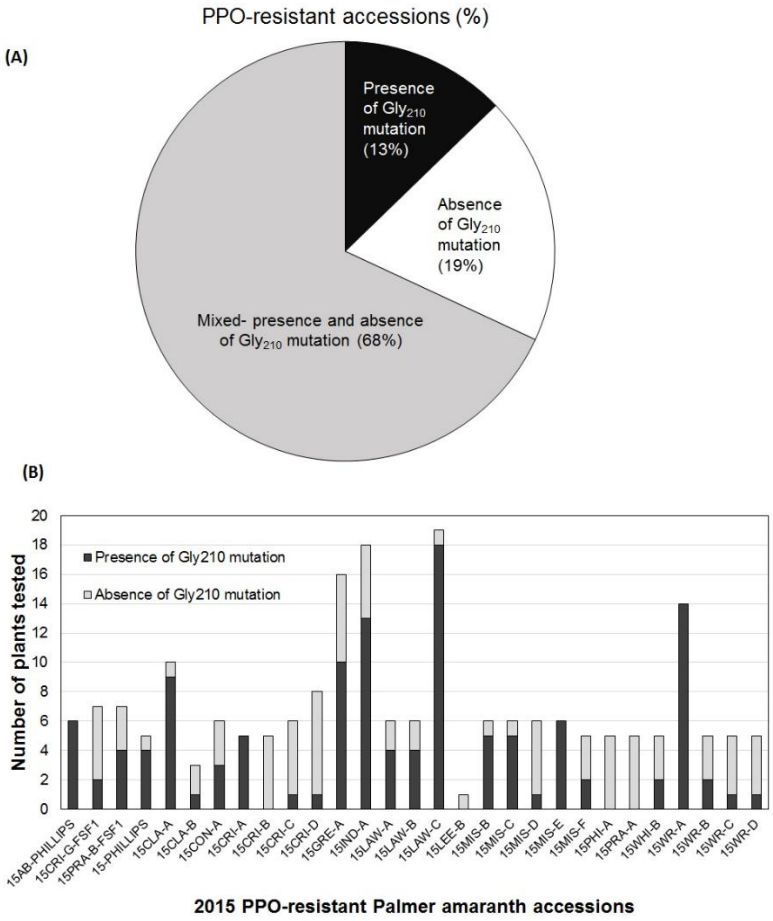

Figure 7. Prevalence of PPO Gly_{210} mutation in PPO-resistant Palmer amaranth from Arkansas. (A) Pie-chart displaying the percentage of PPO-resistant accessions that contained and lacked the PPO Gly_{210} mutation. Black = all fomesafen survivors in these accessions contained the Gly_{210} mutation. White = all survivors in these accessions lacked the Gly_{210} mutation. Gray = survivors in these accessions contained mixture of carriers and non-carriers of Gly_{210} mutation. (B) Proportion of Gly_{210} mutation carriers in each PPO-resistant accession.

CONCLUSIONS

Majority of the Palmer amaranth accessions from Arkansas were sensitive to glufosinate but resistant to trifloxysulfuron and glyphosate. Increased expression of GST and CYPA219 genes were associated with differential tolerance to glufosinate. The elevated expression of these detoxification-related genes can get fixed in population with sustained selection pressure, leading to evolution of resistance. Resistance to ALS inhibitors in a Palmer amaranth population from Arkansas is attributed to ALS $Ser_{653}Asn$ mutation and elevated levels of CYP81B and GSTF10 genes. Induced expression of these non-target genes endow resistance to various ALS herbicide classes.

Palmer amaranth in Arkansas had evolved resistance to PPO-inhibiting herbicides. PPO-resistant Palmer amaranth biotype was first detected in a population collected in 2011. (4) Since then, the number of PPO-resistant populations increased in at least 13 counties in Arkansas in 2015. Fomesafen-resistant populations were generally resistant to trifloxysulfuron and glyphosate and were also cross-resistant to other PPO-inhibiting herbicides such as carfentrazone, flumioxazin, and lactofen. The PPO Gly_{210} deletion is the prevalent (55%) resistance mechanism among PPO-resistant Palmer amaranth populations from Arkansas. Plants carrying PPO Gly_{210} deletion or $Arg_{128}Gly$ mutations had similar level of resistance to fomesafen. Palmer amaranth has multiple genetic weapons, within or outside of the target site, which it can use to counteract the lethal effects of herbicides. Resistance to multiple modes of action, coupled with target and non-target-site mechanisms, severely limits herbicide option for Palmer amaranth control. With Palmer amaranth being an obligate crossing species, plants in a population can accumulate multiple resistance mechanisms which would likely drive resistance evolution faster and is therefore detrimental to chemical weed management.