



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

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

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Plant Material and Growing Conditions

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

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

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

Assessment of TSR Mechanisms in KCTR Palmer Amaranth

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

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

Relative EPSPS Genomic Copy Number Estimation

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

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

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

^aField recommended rate to control Palmer amaranth.^bNonionic surfactant at 0.25% v/v.^cCrop oil concentrate at 1% v/v.^dAmmonium sulfate (34%) at 2% v/v; methylated seed oil at 1% v/v.^eMethylated seed oil at 1% v/v.

were performed with DNA collected from thirteen different KCTR plants that survived glyphosate application as biological replicates, with three technical replicates per DNA sample for both target and endogenous control genes. This experiment was

repeated, and data was combined. To determine the specificity of the qPCR products, a melt curve profile was included following the thermal cycling. The EPSPS copy number in KCTR was estimated using the formula for fold induction ($2^{-\Delta\Delta Ct}$) (Pfaffl, 2001) relative to the reference sample, i.e., glyphosate-susceptible KSS or MSS plants, in each run. The mean EPSPS copy number of KCTR plants along with susceptible KSS and MSS were plotted along with standard error of mean calculated from two experimental runs.

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

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

^aA total of 50 plants from each population (KCTR and KSS or MSS) were sprayed with each herbicide.^bKSS and MSS were controlled ($\geq 95\%$) with all the herbicides.

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

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

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

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

RESULTS

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

Percent survival of KCTR plants to different herbicides was highly variable indicating the considerable genetic variability

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

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

Assessment of TSR Mechanisms in KCTR Palmer Amaranth

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

Assessment of NTSR Mechanisms in KCTR Palmer Amaranth

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

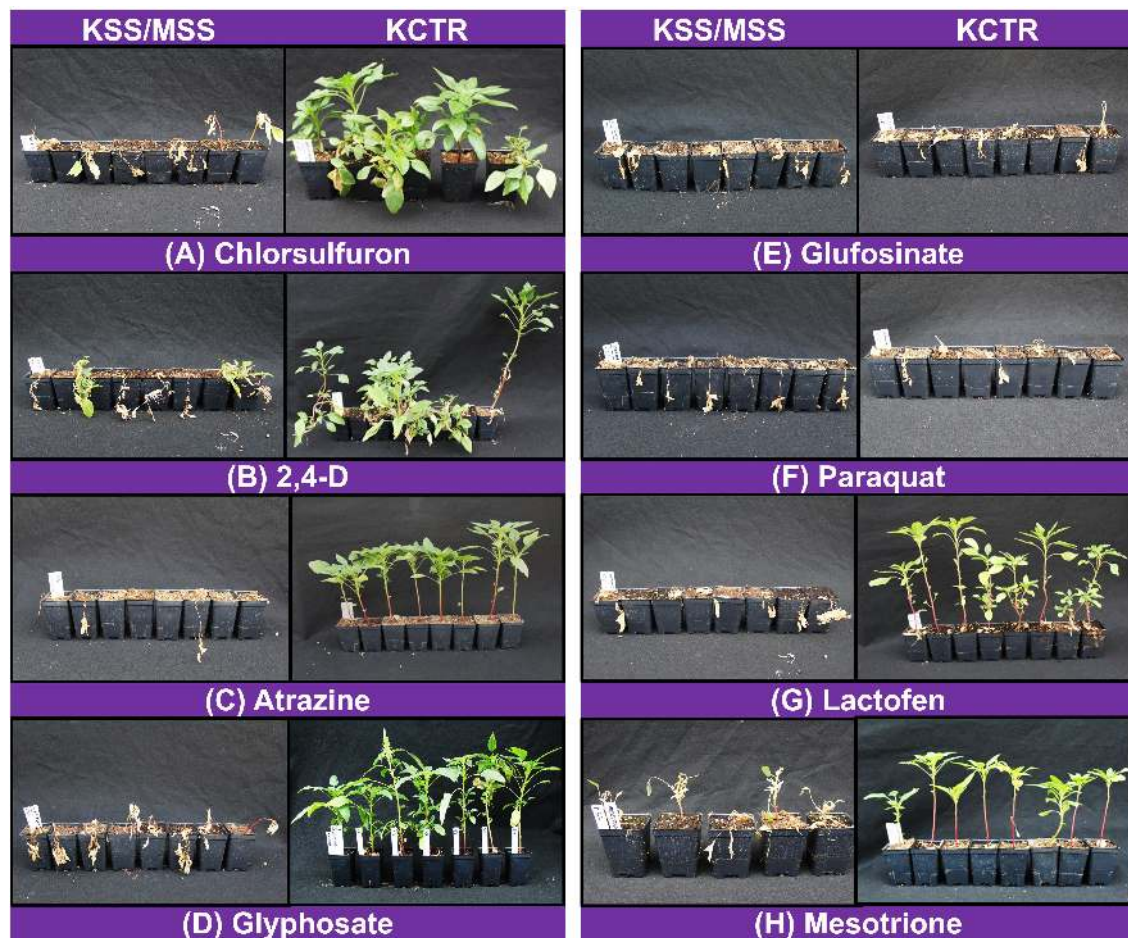


FIGURE 1 | Response of susceptible (MSS or KSS) and resistant (KCTR) Palmer amaranth to chlorsulfuron (A), 2,4-D (B), atrazine (C), glyphosate (D), glufosinate (E), paraquat (F), lactofen (G), and mesotrione (H) application 2–4 weeks after treatment.

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

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

Highly variable biomass accumulation was observed in KCTR plants treated with 2,4-D; however, relative biomass

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

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

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

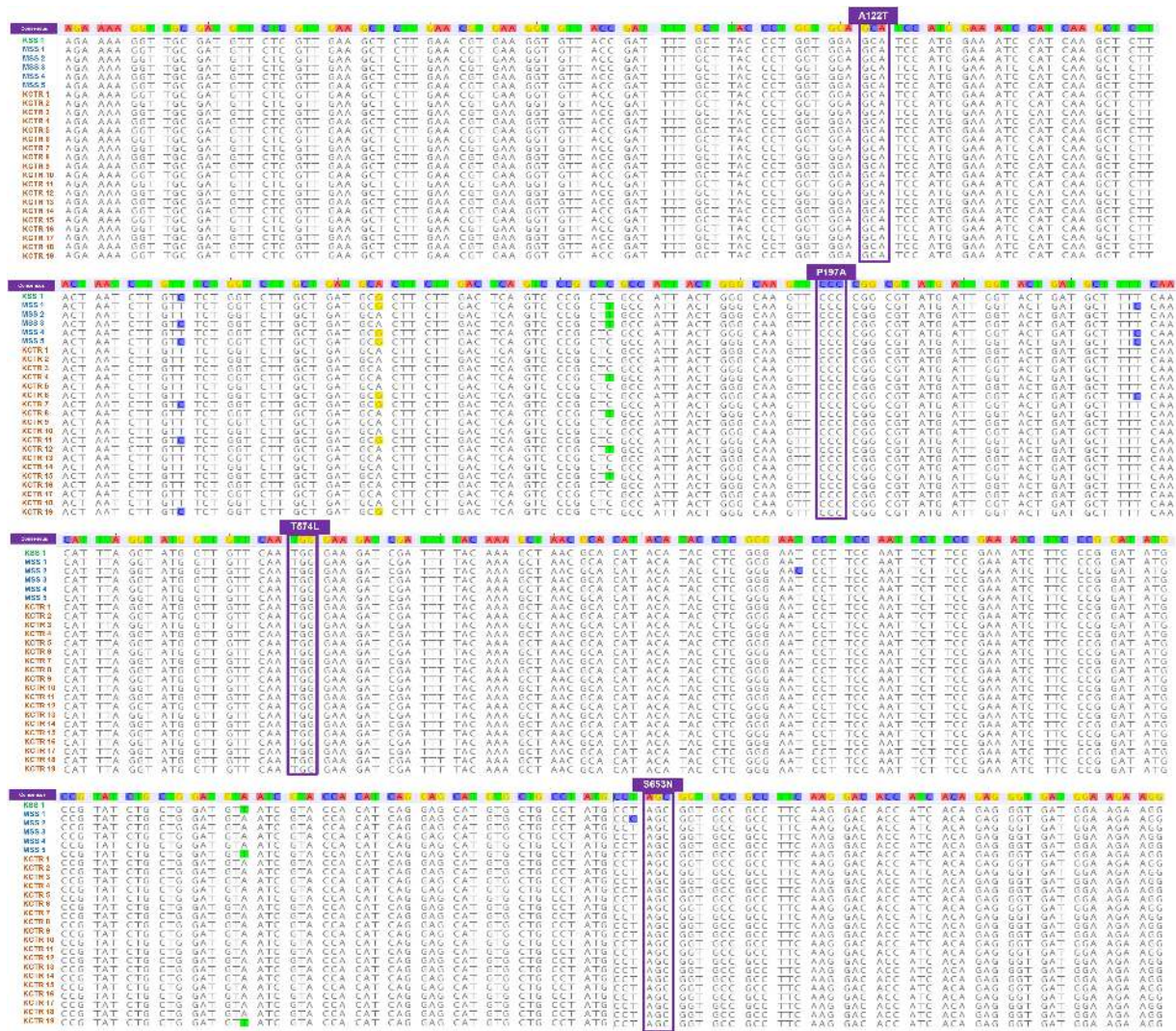


FIGURE 2 | Nucleotide sequence alignment of *ALS* gene fragment from susceptible (MSS and KSS) and chlorsulfuron (KCTR 1–3)- and imazethapyr (KCTR 4–19)-resistant Palmer amaranth. Nucleotide polymorphisms were observed across KCTR, KSS, and MSS Palmer amaranth, but no amino acid substitutions were found.

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

DISCUSSION

Palmer amaranth is a dioecious species with prolific seed production that has already evolved resistance to eight herbicide SOAs (Heap, 2020). In this research, we report for the first time the evolution of six-way resistance in a single Palmer amaranth population, i.e., KCTR with predominance of metabolic resistance mechanisms. Previously Palmer amaranth

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

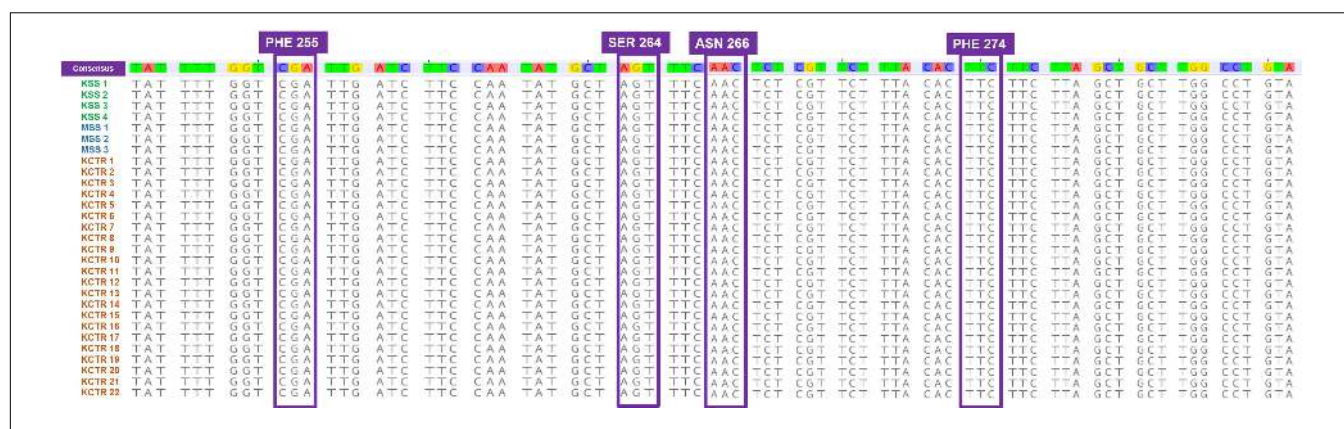


FIGURE 3 | Nucleotide sequence alignment of *psbA* gene fragments from atrazine-susceptible (MSS and KSS) and -resistant (KCTR) Palmer amaranth individuals. No nucleotide polymorphisms were observed among KCTR, KSS, and MSS Palmer amaranth individuals.

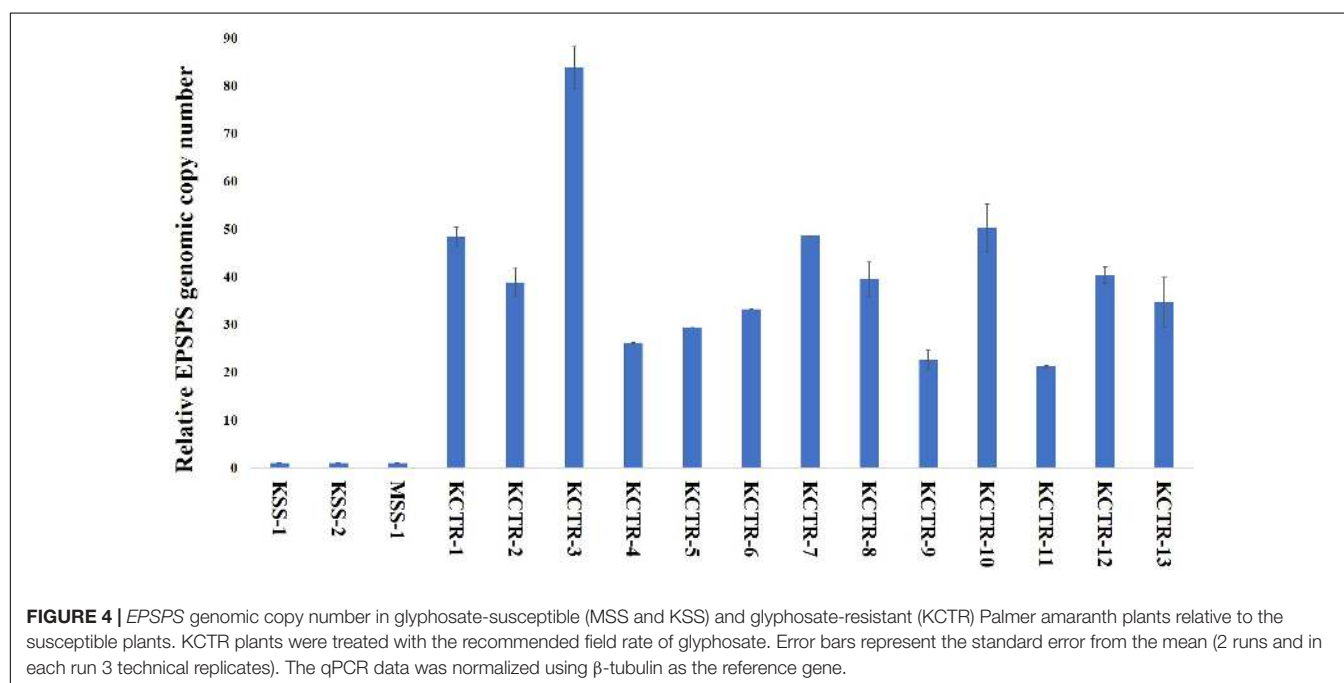
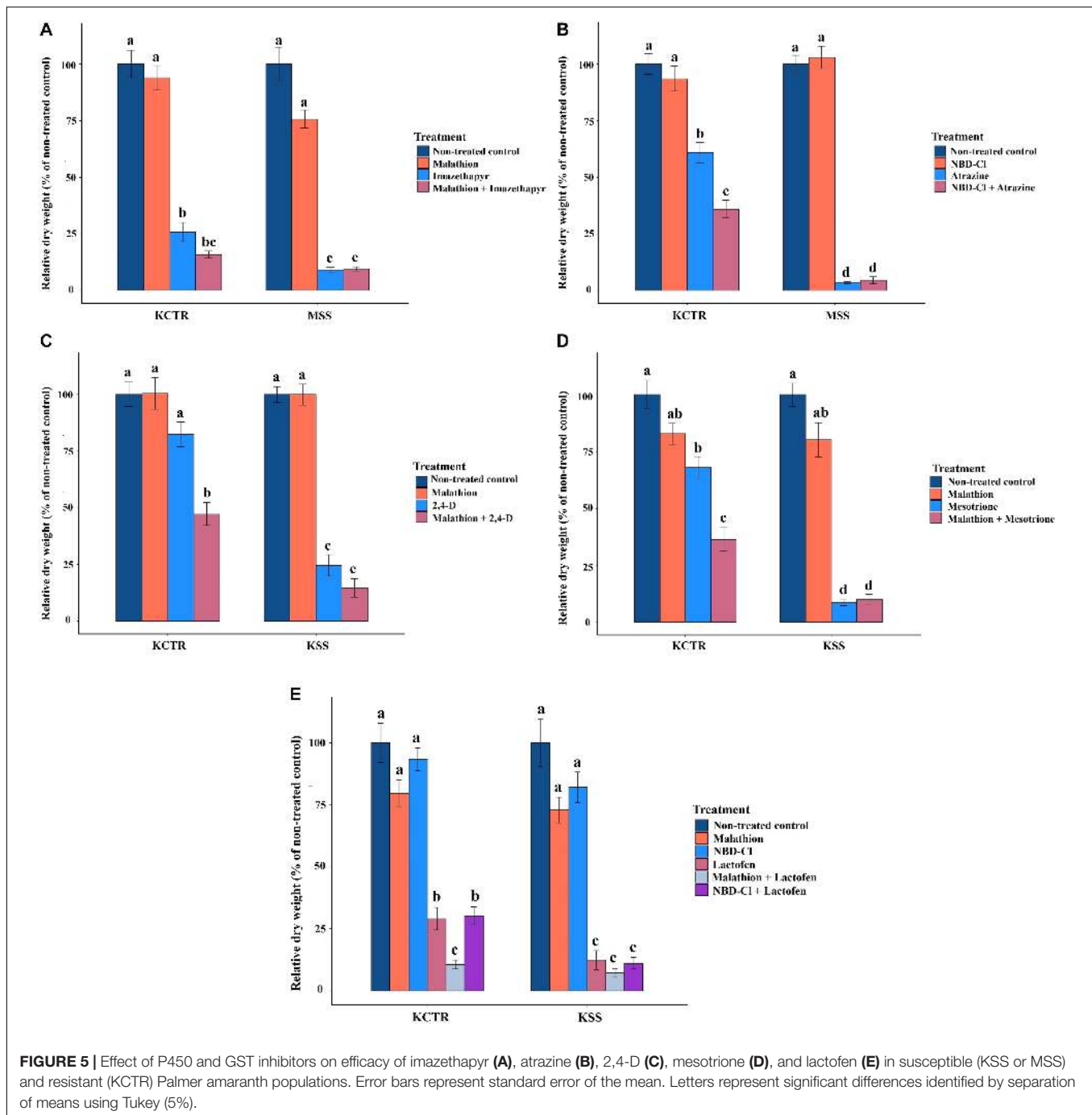


FIGURE 4 | EPSPS genomic copy number in glyphosate-susceptible (MSS and KSS) and glyphosate-resistant (KCTR) Palmer amaranth plants relative to the susceptible plants. KCTR plants were treated with the recommended field rate of glyphosate. Error bars represent the standard error from the mean (2 runs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

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

Although mutations in herbicide target genes conferring resistance are rare evolutionary events, mutations in the *ALS* gene at eight positions (four positions in Palmer amaranth) were found to confer resistance to several classes of ALS

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



reduction of biomass in KCTR with malathion treatment. Moreover, since only 60% of KCTR plants were found to be resistant to imazethapyr (Table 2), it is possible that sensitive plants in KCTR may have contributed to the lack of differences between imazethapyr only and combination of malathion with imazethapyr treatment. Different P450 inhibitors have been observed to have varying levels of synergistic effect in resistant weeds depending on the herbicide as well as P450 isozyme. For instance, Preston et al. (1996) observed piperonyl butoxide (PBO; another P450 inhibitor) inhibiting

chlorotoluron and simazine resistance in rigid ryegrass (*Lolium rigidum*), but malathion failed to do so. Similarly, Oliveira et al. (2018) observed that malathion improved the efficacy of tembotrione but not of mesotrione in HPPD-inhibitor-resistant common waterhemp from Nebraska. Therefore, it is possible that different P450s, which are not completely inhibited by malathion, are involved in imparting resistance to imazethapyr in KCTR.

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

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

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

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

Based on previous research in our laboratory, the coexistence of both TSR and NTSR for the same herbicide can occur in a single population or individual plant of Palmer amaranth (Nakka et al., 2017a,b; Chaudhari et al., 2020). Research is in progress to determine if such a scenario is present in KCTR as well. Future investigations focused on identifying specific P450s and GSTs involved in herbicide detoxification in this population

will also be investigated. It is important to understand what predisposes a population to develop metabolic resistance to several SOAs, including those with no history of use and absence of selection pressure.

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

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

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

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

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

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