PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF MULTIPLE HERBICIDE RESISTANCE IN PALMER AMARANTH (*AMARANTHUS PALMERI*)

by

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M.S., Acharya N.G. Ranaga Agricultural University, 2005
M.S., University of Illinois, 2008

AN ABSTRACT OF A DISSERTATION

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Abstract

Palmer amaranth (*Amaranthus palmeri*) is one of the most aggressive, troublesome and damaging broadleaf weeds in many cropping systems including corn, soybean, cotton, and grain sorghum causing huge yield losses across the USA. As a result of extensive and intensive selection of pre- and -post emergence herbicides, Palmer amaranth has evolved resistance to multiple herbicide modes of action, microtubule-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, acetolactate synthase (ALS)-, photosystem II (PS II)-, hydroxyphenylpyruvate dioxygenase (HPPD)- and more recently to protoporphyrinogen oxidase (PPO)-inhibitors. A Palmer amaranth population from Kansas was found resistant to HPPD-, PS II-, and ALS-inhibitors. The overall objective of this research was to investigate the target-site and/or non-target-site resistance mechanisms in Palmer amaranth from KS (KSR) to mesotrione (HPPD-inhibitor), atrazine (PS II-inhibitor), and chlorsulfuron (ALS-inhibitor) relative to known susceptible Palmer amaranth from Mississippi (MSS) and KS (KSS). Whole plant dose-response assays showed high level of resistance in KSR to mesotrione, atrazine and chlorsulfuron. KSR was 10-18, 178-237 and >275 fold more resistant to mesotrione, atrazine, and chlorsulfuron, respectively, compared to MSS and KSS. Metabolism studies using [^{14}C] labeled mesotrione and atrazine demonstrated non-target-site resistance to both herbicides, particularly, enhanced metabolism of [^{14}C] mesotrione likely mediated by cytochrome P450 monooxygenases and rapid degradation of [^{14}C] atrazine by glutathione S-transferases (GSTs). In addition, molecular and biochemical basis of mesotrione resistance was characterized by quantitative PCR (qPCR) and immunoblotting. These results showed 4-12 fold increased levels of the *HPPD* transcript and positively correlated with the increased HPPD protein. Sequencing of atrazine and chlorsulfuron

target genes, *psbA* and *ALS*, respectively, showed interesting results. The most common mutation (serine264glycine) associated with atrazine resistance in weeds was not found in KSR. On the other hand, a well-known mutation (proline197serine) associated with chlorsulfuron resistance was found in 30% of KSR, suggesting ~70% of plants might have a non-target-site, possibly P450 mediated metabolism based resistance. Over all, KSR evolved both non-target-site and target-site based mechanisms to mesotrione and chlorsulfuron with only non-target-site based mechanism of resistance to atrazine leaving fewer options for weed control, especially in no-till crop production systems. Such multiple herbicide resistant Palmer amaranth populations are a serious threat to sustainable weed management because metabolism-based resistance may confer resistance to other herbicides and even those that are yet to be discovered. The findings of this research are novel and valuable to recommend appropriate weed management strategies in the region and should include diversified tactics to prevent evolution and spread of multiple herbicide resistance in Palmer amaranth.

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Dedication

This dissertation is dedicated to my two beautiful daughters, Dharitri and Sahasra.

Chapter 1 - Literature Review

History and Significance of Herbicides

In the past century, the global food production has increased substantially, and this increase has been attributed to the development of high-yielding crop varieties, use of fertilizer and pesticides, and increased land under cultivation, etc. However, agricultural production across the world is significantly impacted by crop losses due to incidence of pests and natural disasters. These losses include damage caused by insects, nematodes, mites, pathogens, and weeds. Substantial crop failure in particular, because of weed infestation accounts for the highest economic loss up to 34% globally (Oreke 2006). In the USA alone, economic loss due to weeds is estimated more than $26 billion annually (Pimentel et al., 2000). Successful crop protection measures such as the use of insecticides and fungicides have been adopted as early as the 1900s. However, the necessity for the use of herbicides in agriculture, especially in the United States was not known until the labor for hand weeding became inadequate and more expensive with an increase in agriculture acreage (Gianessi and Reigner 2007). Currently, the use of herbicides has become indispensable for weed management in both crop and non-crop areas.

According to Weed Science Society of America (WSSA), in the United States, the economic loss due to weeds, in the absence of herbicide use was estimated at $15.5 billion per year (Bridges 1992). Recently, these economic losses in crop yields have increased to billions of dollars in a single crop with potential yield losses of more than $26 and >$15 billion in corn and soybean cropping systems, respectively, when no herbicides are used to manage weeds during crop production (Dille 2015 and 2016). Advancement in agricultural practices have increased no-till acreage in crop production to reduce land degradation, prevent soil erosion, conserve soil moisture and increase crop productivity (Kihara 2012). However, no-till conditions are favorable

for the growth of many weeds such as kochia (*Kochia scoparia*), marestail (*Conyza canadensis*), gaint ragweed (*Ambrosia trifida*), Palmer amaranth (*Amaranthus palmeri*), and waterhemp (*Amaranthus tuberculatus*), *etc*. Hence, no-till agriculture depends heavily on herbicide application for weed control. Consequently, extensive and intensive use of herbicides has resulted in the evolution and spread of herbicide-resistant weeds. According to the International survey of herbicide resistant weeds, 249 species (144 dicots and 105 monocots) have evolved resistance to 23 of the 26 known herbicide sites/modes of action since the evolution of the first case of herbicide-resistant weed in 1957 (Heap 2016). Kochia, marestail, waterhemp and Palmer amaranth are the most important and economically troublesome weeds of the US Midwestern agriculture and have evolved resistances to one or more herbicides which are commonly used in agriculture. The focus of this dissertation is on the evolution of multiple herbicide resistance in Palmer amaranth, which is discussed in more detail hereunder.

Origin and Distribution of Palmer Amaranth

Palmer amaranth is native to Southern part of USA in California, New Mexico and Texas (Sauer 1957) and has the long history of significance as being edible and highly nutritious. Palmer amaranth and other closely related species have been grown for thousands of years for their leaves and seeds in Mexico, South America, the Caribbean, Africa, India, and China. Native American Tribes, Navajo, Pima, Yuma, and Mohave, have used Palmer amaranth leaves as greens and seed as grain (Moerman 1998). Distribution of Palmer amaranth beyond its native habitat occurred through migration of human beings to new habitats, dispersal of the seed through wind and water. It has become one of the thriving plants in diverse environmental conditions

Biological Characteristics of Palmer Amaranth

Palmer amaranth belongs to the family Amaranthaceae. There are about 75 *Amaranthus* species in the family and Palmer amaranth is one of ten dioecious species in the genus. All ten dioecious species are native to North America with the rest of the monoecious *Amaranthus* species distributed across the world (Steckel 2007). The genus name *Amaranthus* is derived from the Greek word 'amarantus' meaning 'everlasting' or 'never failing flower'. Palmer amaranth is also called as a careless weed, dioecious amaranth, Palmer's amaranth or Palmer's pigweed. Morphologically, Palmer amaranth has green to reddish stems or striated colors with alternate leaves and petioles often longer than leaf blade (Sauer 1955). The leaves have V-shaped variegation on the upper surface similar to the monoecious spiny amaranth (Franssen et al., 2001). Male and female inflorescences are present as terminal spikes of up to 30-60 cm long on male and female plants, respectively. Lateral stems also produce inflorescences but are shorter than terminal inflorescence. Palmer amaranth is an obligate outcrossing species due to its dioecious nature, and each female plant can produce from 200,000-600,000 seeds under ideal growth conditions and no competition (Keeley et al., 1987).

Palmer amaranth is a C_4 dicot with high rates of photosynthesis (81 µmol m^{-2} s^{-1}) compared to other C_4 monocots such as corn and C_3 dicots like cotton and soybean (Ehleringer 1983; Gibson 1998). The high photosynthetic rates enable Palmer amaranth grow rapidly with more than 5 cm per day under bright sunlight and ideal conditions (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). More importantly, Palmer amaranth exhibits diaheliotropism, meaning the plant is capable of tracking the sunlight (solar tracking) where the leaves orient themselves perpendicularly to the sun rays to maximize the light interception and thus photosynthesis (Ehleringer and Forseth 1980).

Palmer Amaranth as A Weed

Palmer amaranth is a summer annual broadleaf weed and biological attributes such as high rates of photosynthesis, aggressive growth habit, prolific seed production, extended periods of germination and adaptation to diverse environmental conditions makes it a successful and competitive weed in many cropping systems in the United States. Palmer amaranth competes for sunlight, water, and nutrients resulting in huge yield losses depending on its density and the duration of interference during crop growth. When the density of Palmer amaranth increased from 0.5-8 plants m^{-1} of corn row, the crop yield was reduced by 11-91% (Massinga et al., 2001). Season-long competition of Palmer amaranth with sorghum reduced the yields (up to 38-63%) and affected the harvest efficiency (Moore et al., 2005). Similarly, Palmer amaranth infestation at a density of 10 plant m^{-1} row resulted in 68% of soybean yield loss (Klingaman and Oliver 1994). In a different study, the season-long interference of Palmer amaranth at 8 plants m^{-1} row reduced the soybean yield by 78% (Bensch et al., 2003). In soybean, when the competitiveness of three *Amaranthus* species, Palmer amaranth, waterhemp and redroot pigweed (*A. retroflexus*) at densities of 0.25-4 plants m^{-1} row was evaluated, Palmer amaranth accumulated more biomass, produced more seed and caused more soybean yield loss than the other two weed species (Bensch et al., 2003). Likewise, Palmer amaranth infestation decreased biomass of cotton crop by more than 50%, and 8 weeks after emergence and season-long interference reduced the cotton yields from 13-54% (Morgan et al., 2001).

In other crops, e.g. peanut, Burke et al. (2007) reported the yield losses of 28 and 68% due to season-long interference of Palmer amaranth at 1 and 5.5 plants m^{-1} row, respectively. The quality of sweet potatoes was also reduced by 56 and 94% at a density of 0.5 and 6 Palmer amaranth plants m^{-2} (Meyers et al., 2010). Competitive interference of Palmer amaranth in crops affects the harvest efficiency in addition to quantity and quality of the yield (Smith et al., 2000).

Besides competitive interference, allelopathic effects of Palmer amaranth were also known to affect the seedling growth of grain sorghum, cabbage, carrot, onion and tomato (Menges 1988; Connick et al., 1987).

Herbicide Selection Pressure and Evolution of Resistance to Herbicides

Repeated and widespread use of herbicides to control weeds exerts a strong selection pressure and ultimately results in the evolution and spread of herbicide-resistant weeds. According to WSSA, herbicide resistance is defined as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type". The herbicide-resistant weeds can be grouped into three types: a) single-, b) cross-, and c) multiple-resistant. Resistance to a single mode of action of herbicide is called single herbicide resistance. Cross-resistance is defined as resistance to two or more herbicide classes with the same mechanism of action, while multiple resistance is resistance to two or more herbicides with different mechanisms of action. Weed populations naturally have individual plants that are resistant to herbicides, regardless of herbicide application (Jasieniuk et al., 1996; Gasquez 1997). However, the resistant individuals exist at very low frequencies and are difficult to identify from susceptible individuals in the population. Repeated applications of herbicides with the same mode of action select the herbicide-resistant individuals that survive and reproduce. Continuous use of the herbicide results in the rapid spread of the resistant individuals and eventually become dominant replacing the susceptibles in the population.

Besides selection pressure of herbicides, other factors such as biological characteristics of weed species, genetic factors, characteristics of herbicides and agronomic practices also play an important role in the evolution and spread of herbicide resistance in weed species (Powles and Yu 2010). The characteristics of weeds that facilitate the evolution of herbicide resistance

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include high fecundity of individual plants producing thousands of seeds, high germination percentage, wide window of emergence, seed dispersal and short longevity and high frequency of resistant individuals in the population. The genetic factors which influence the evolution of herbicide resistance include natural mutations conferring herbicide resistance, frequency, and dominance of resistant genes in the weed population and fitness cost of resistance genes in the presence or absence of herbicide application (Jasieniuk et al., 1996; Délye et al., 2013). When the initial frequency of resistant genes is high, there will be a rapid development of resistance. On the other hand, if the resistant genes are rare in the weed population, then the evolution of resistance may take several years or may not develop at all. The herbicide characteristics such as structural properties, site/mode of action and residual activity of herbicide and agronomic practices such as applying lower dose than recommended field rates can influence the development of herbicide resistance. Additionally, the time of herbicide application, i.e. the size of weeds, climatic conditions, limited or no crop rotation also play a critical role in the evolution of resistance to herbicides. Recent advancements in agronomic practices have increased no-till and reduced tillage acreage in crop production to prevent soil erosion and for several other advantages (Kihara 2012). However, adoption of no-till agricultural production heavily depends on herbicides for weed management, and thus, the herbicide selection results in the evolution of herbicide-resistant weeds.

Mechanisms of Herbicide Resistance

Herbicides have physical and chemical properties that allow them to be absorbed and translocated to reach the target-site resulting in the injury and death of a susceptible weed. However, when a weed is resistant to a herbicide, the herbicide is not lethal any more due to: a) changes in the target-site or b) detoxification or sequestration during the process of absorption

and translocation before reaching the target site (Powles and Holtum 1994; LeBaron and Gressel 1982; DePrado et al., 1997; Caseley et al., 1991). Thus, herbicide resistance mechanisms are broadly classified into two types: target-site resistance mechanism and non-target-site resistance mechanism.

Target-Site Resistance Mechanisms

Evolution of target-site resistance in weeds can be conferred by a point mutation resulting in an amino acid substitution, over expression and/or amplification of the herbicide target gene or through regulatory changes (in promoter) in the target-site. An alteration through a point mutation in the target-site prevents the binding of herbicide to the target and amplification or increased expression of the herbicide target gene can produce more target protein without affecting the normal functioning of the plant. Target-site based herbicide resistance mechanisms are more common to certain herbicide modes of action than others, such as acetyl CoA carboxylase (ACCase)-, acetolactate synthase (ALS)-, and photo system II (PS II)-inhibitors due to the flexibility of protein structure to function normally along with preventing the herbicide binding.

ACCase-inhibitors belong to lipid synthesis-inhibitors and biochemically inhibit the ACCase enzyme which primarily catalyzes the first reaction in the *de novo* synthesis of fatty acids (Secor and Cséke 1988). There are three chemical classes of ACCase herbicides, namely cyclohexanediones, commonly called as DIMS, aryloxyphenoxypropionates, as FOPS and phenyl pyraxolines, as DENS (Hofer et al., 2006). These herbicides are commonly called graminicides because of their selective activity on grasses and are widely used in broadleaf crops (Konishi and Sasaki 1994). Many grass weeds evolved resistance to ACCase herbicides via target-site resistance mechanism. Eight single amino acid substitutions at seven ACCase codons resulting in isoleucine1781leucine, tryptophan1999cysteine, tryptophan2027cysteine, isoleucine2041asparagine, isoleucine2041valine, aspartate2078glycine, cysteine2088arginine,

glycine2096alanine have been reported (Délye 2005; Powles and Yu 2010). The amino acid

substition of isoleucine1781leucine was found to be most common and was reported in black

grass (*Alopecurus myosuroides*) wild oat (*Avena fatua, A. sterilis*), rigid ryegrass (*Lolium

rigidum, L. multiflorum*), and green foxtail (*Setaria viridis*) (Petit et al., 2007; White et al., 2005;

Christoffers and Pederson 2007; Powles and Yu 2010). Populations of ryegrass and blackgrass

have shown most of the other mutations except tryptophan1999cysteine. However, the level of

resistance conferred by each mutation varies (Délye et al., 2008; Yu et al., 2007).

Mutations as well as amplification of the target gene have also been reported for 5-

enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibior (e.g. glyphosate). Glyphosate

competitively inhibits the EPSPS enzyme, affecting the biosynthesis of aromatic amino acids,

phenylalanine, tryptophan and tyrosine (Jaworski 1972). Glyphosate is the most widely used

herbicide and controls broad spectrum of annual and perennial weeds. The first case of

glyphosate resistance was reported in rigid ryegrass (Powles et al., 1998; Pratley et al., 1999) and

then in goosegrass (*Eleusine indica*) due to the proline106serine substitution (Lee and Ngim

2000; Baerson et al., 2002). Other amino acid substitutions, threonine and alanine at proline106

position of *EPSPS* gene have also been reported in goosegrass and ryegrass populations,

respectively (Ng et al., 2003; Yu et al., 2007). Recently, a double amino acid substitution,

threonine102isoleucine and proline106serine (TIPS) in the *EPSPS* gene has been reported to

confer a high level of resistance to glyphosate in goosegrass (Yu et al., 2015). On the other hand

amplification of *EPSPS* gene (>100 sopies) has been first reported in glyphosate-resistant Palmer

amaranth with a 40-fold increase in EPSPS expression compared to the susceptible biotype

(Gaines et al., 2010). Subsequently, *EPSPS* gene amplification is reported in many other

glyphosate-resistant weed species such as Italian ryegrass, kochia, and spiny amaranth

(*Amaranthus spinosus*) (Salas et al., 2012; Wiersma et al., 2015; Varanasi et al., 2015; Nandula et al., 2014). The target-site based resistance is also common in PS II- and ALS-inhibitor-resistant weeds, which is discussed in more detail later in this chapter.

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Non-Target-Site Resistance Mechanisms

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Non-target-site herbicide resistance involves one or a combination of several mechanisms that limit the quantity herbicide reaching the target-site. These mechanisms include reduced absorption, decreased translocation or enhanced metabolism of herbicides. Crop tolerance and selectivity to herbicides is known to occur because of metabolism of herbicides in corn, sorghum, wheat. (Cole 1994; Deprado et al., 2012; Hatton et al., 1996; Kreuz et al., 1991; Lamoureux et al., 1973; Shimabukuro et al., 1971 Two enzyme families, cytochrome P450 monoxygenases and glutathione S-transferases (GSTs) are known to be involved in rapid detoxification of herbicides.

Herbicide Resistance as a Result of Reduced Absorption and Translocation

Herbicide absorption can occur through leaves, shoots or roots. Absorption by roots and leaves is the major mode of soil and foliar applied herbicides, respectively. Systemic herbicides once inside the root or leaf are translocated in the vascular system of the plant to reach the target-site. Absorption of the herbicides is enhanced by the addition of surfactants (non ionic surfactant, NIS) and adjuvants such as crop oil concentrate (COC), methylated seed oil (MSO), ammonium sulfate (AMS) which dissolve the epicuticular waxes and increase the spray droplet coverage and retention. Weeds can evolve mechanisms to reduce absorption and/or translocation of the herbicide. Many populations of horseweed and ryegrass were reported to have reduced translocation of glyphosate contributing to resistance (Wakelin et al., 2004; Feng et al., 2004; Preston and Wakelin 2008). Glyphosate-resistant johnsongrass (*Sorghum halepense*) populations

also showed reduced uptake and decreased translocation (Vila-Aiub et al., 2012). Some ryegrass populations had both mutation in *EPSPS* gene resulting in the substitution of proline106serine as well as restricted translocation of herbicide (Preston et al., 2009).

Metabolism Based Herbicide resistance

Herbicides can act as substrates for the enzymes involved in the detoxification process, and the process is carried out by specific enzyme families such as cytochrome P450 monooxygeneses and glutathione S transferases. Often, the herbicide is metabolized before reaching the target-site which is usually the common mechanism of tolerance to many herbicides in crop species.

Cytochrome P450 monoxygenases

Cytochrome P450s are heme-containing monooxygenases involved in both biosynthetic and detoxificaton pathways (Chapple 1998; Schuler 1996) and are present across all kingdoms. Cytochrome P450s are also known as mixed or multi-functional oxidoreductases which use NADPH and/or NADH to cleave oxygen molecule to form an organic substrate with a functional group and water. Some P450s are tissue and substrate specific, and regulated at individual loci in the genome. However, many different cytochrome P450s can act on a single substrate, and a single P450 can have many different substrates (Lupien et al., 1999; Haudenschild et al., 2000; Tijet et al., 1998; Le Bouquin et al., 2001). Cytochrome P450s belong to cytochrome b type (a, b and c) and are characterized by their absorption spectrum around 420 nm of visible light (Omura 1993). Cytochrome P450s differ in their coding sequences, intron positions and upstream regulatory elements among and within organisms. The cytochrome P450s are classified into different families and sub-families depending on the percent amino acid identity (Nelson et al., 1996). The enzyme families are grouped into CYP1, CYP2, CYP3, etc. The members that

represent these families share more than 40% amino acid identity. The sub-families are categorized with letters as CYP1A, CYP1B, CYP1C, etc with members having greater than 55% amino acid identity. Sub-families are again grouped as CYP1A1, CYP1A2, CYP1A3, etc based on the individual loci. This is a highly structured nomenclature of cytochrome P450s and it is followed universally to study functions and relationships between proteins in an organism as well as across the kingdoms.

Herbicide metabolism and detoxification in plants can occur in three phases. Phase I conversion includes reactions such as oxidations, reductions and hydrolysis and Phase II metabolism involves conjugation of herbicides with glucose, amino acids or glutathione (GSH) and while Phase III detoxification results in the conversion of the primary conjugates to secondary conjugates which gets deposited into the vacuoles or other compartments (Hatzios 1997). Cytochrome P450 monoxygenases and esterases are involved in Phase I conversion of insecticides, herbicides and some pollutants, leading to crop tolerance, insecticide and herbicide resistance to these compounds (Cole 1994; Siminszky 2006). Specific examples of herbicides which are metabolized by P450s include: chlorotoluron in wheat, corn and cotton (Moughin et al., 1992), bentazoan in soybean (Sterling and Balke 1990), fenoxaprop-p-ethyl in barley (Romano et al., 1993), clomazone in cotton (Ferhatoglu et al., 2005), pyrazosulfuron ethyl in rice (Yun et al., 2001), clodinafop in wheat, barley, and corn (Kreuz et al., 1991), thiazopyr in many weedy species (Feng et al., 1995), and mesotrione in waterhemp (Ma et al., 2013).

When a cytochrome P450 metabolizes an herbicide, a typical reaction results in the formation of a hydroxylated metabolite of the herbicide which is less toxic and further catalyzed through Phase II conjugation or Phase III compartmentation (Shimaburko 1985). It has been shown that chlorotoluron is metabolized by P450s through oxidative N-demethylation and

hydroxylation of the ring-methyl group (Gonneau et al., 1988) in wheat and barley. In *Echinocloa phyllopogan*, a common weed in rice evolved resistance to bispyribac-sodium, fenoxaprop-ethyl and thiobencarb through induction and detoxification by cytochrome P450s (Yun et al., 2005). Specific cytochrome P450's, CYP72A31 and CYP81A6 are involved in the metabolic resistance of two ALS-inhibitors, bispyribac sodium and bensulfuron-methyl in rice and *Arabidopsis*. In rigid ryegrass, four putative P450s: CYP72A, CYP716A, CYP71B and CYP89A that are involved in the resistance to diclofop, an ACCase-inhibitor are identified by RNA-Seq transcriptome analysis (Gaines et al., 2014).

Among all the known resistance mechanisms, cytochrome P450 mediated metabolism of herbicides presents a threat to sustainable agricultural production because the P450 monoxygenases are capable of detoxifying many herbicides with different modes of action including the herbicides which are never used for weed control and even those that are yet to be discovered.

Glutathione S-Transferases (GST)

Similar to cytochrome P450 monoxygenases, GSTs are multifunctional enzymes involved in normal plant growth and development processes, cellular metabolism and detoxification of xenobiotics (Marrs 1996). GSTs catalyze the transfer of a tripeptide, γ-glutamyl-cysteinyl-glycine known as glutathione (GSH) to electrophilic reaction center of many substrates of endogenous and exogenous origin (Mannervik et al., 1988). In plants, GSTs are encoded by a large diverse gene family and classified into three different types based on the amino acid sequence identity and the intron:exon placement (Droog et al., 1995; Edwards et al., 2000). Type I GSTs have three exons and two introns and include enzymes involved in herbicide detoxification and stress defense like oxidative stress from wounding and pathogen attack. These

GSTs also respond to plant hormone, auxin. Type II GSTs contain ten exons and nine introns and respond to ethylene and senescence. They are homologous to GSTs in mammals. Type III GSTs have two exons and one intron and induced by auxins, heat shock, pathogen infection and heavy metals.

The significance of GSTs in herbicide detoxification was documented in 1970 when atrazine was conjugated with GSH by a maize GST, protecting the crop from herbicide injury (Shimabukuro et al., 1971). Since then, many GSTs were isolated and characterized for their role in the herbicide tolerance, selectivity, resistance and induction in many crops and weed species (Marrs 1996; Cole et al., 1997). GSTs are known to metabolize and detoxify atrazine, alachlor, metolachlor, fluorodifen, chloroacetanilide, and thiocarbamates. Corn and sorghum contain high levels of GSTs and provide natural tolerance to triazines, alachlor, metolachlor, and EPTC (Shimabukuro et al., 1971; Lamoureux et al., 1973; O'Connell et al., 1988; Lay and Casida 1976). Many susceptible crops like wheat, barley, peas, and several broadleaf weeds do not have GST activity (Shimabukuro et al., 1977). However, these crops can tolerate the herbicides when used with safeners. Safeners are compounds that enhance herbicide tolerance in grass crops by activating the pathways of xenobiotic detoxification via GST conjugation. Fenchlorazole-ethyl is an herbicide safener and when used with dimethenamid and fenoxaprop in wheat, the tau class of GSTs (GSTU) are induced to confer tolerance to these herbicides (Cummins et al., 1997). In rice, increased GST activity in roots and shoots was observed when pretilachlor herbicide was treated with fenclorim safener (Deng and Hatzios 2002). Similarly, there are many safeners that are used with herbicides to protect the crops from herbicide injury by inducing GSTs.

Many broadleaf weeds are resistant to atrazine due to mutation in target-site. However, similar to corn and sorghum, the broadleaf weeds such as velvetleaf and waterhemp evolved

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resistance to atrazine via GST based metabolism (Anderson and Gronwald 1991; Ma et al., 2013). Likewise, many grass weeds also evolved resistance to atrazine by GST-mediated metabolism. In blackgrass, both GSTs and P450s are implicated in the multiple herbicide resistance. It was found that the levels of type I GST, *Am*GST2 was elevated in multiple herbicide-resistant blackgrass compared to the susceptible biotype. Interestingly, the activity of *Am*GST2 was limited in detoxifying herbicides but had high activity as a glutathione peroxidase, scavenging the hydrogen peroxides formed due to herbicide stress (Cummins et al., 1999). Similarly, watergrass was resistant to fenoxaprop-*p*-ethyl due to rapid GHS-conjugation of the herbicide (Bakkali et al., 2007).

Evolution and Mechanisms of Herbicide Resistance in Palmer amaranth

Palmer amaranth has evolved resistance to six different herbicide modes of action across United States including microtubule-, photosystem II (PS II)-, acetolactate synthase (ALS)-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, hydroxyphenylpyruvate dioxygenase (HPPD)- , and more recently to protoporphyrinogen oxidase (PPO)-inhibitor herbicides (Heap 2016). Some Palmer amaranth populations across the United States were also found resistant to multiple herbicides. A comprehensive discussion of evolution of resistance to above herbicides in Palmer amaranth is presented below.

Microtubule-Inhibitor Resistance

Microtubule-inhibitors (e.g. trifluralin, pendimethalin) kill the susceptible plants by inhibiting the mitosis via binding to the tubulin, a protein required for microtubule formation during cell division. Palmer amaranth resistance to trifluralin was confirmed in South Carolina in 1989 from fields where cotton was a major crop. Varying levels of resistance to dinitroaniline herbicides (e.g benefin, isopropalin, pendimethalin, and ethalfluralin) was reported in Palmer

amaranth populations (Gossett et al., 1992). Trifluralin-resistant Palmer amaranth was also reported later in Tennessee in 1998. The mechanism of trifluralin resistance in Palmer amaranth is unknown.

PS II-Inhibitor Resistance

Atrazine resistance in Palmer amaranth was first documented in Texas in 1993 and later in KS (1995), GA (2008) and more recently in NE (2011). The mechanism of resistance to atrazine as a result of serine264glycine mutation was reported in *Amaranthus* species, waterhemp (Matthew et al., 1998) and Powell amaranth (*A. powellii*) (Diebold et al., 2003). However, other populations of waterhemp evolved resistance to atrazine via GST-mediated conjugation (Ma et al., 2013). However, the mechanism of atrazine resistance is not known in Palmer amaranth.

ALS-Inhibitor Resistance

ALS-inhibitor resistant Palmer amaranth was first documented in 1993 in KS (Horak and Peterson 1995) and thereafter reported in many states across the US (Heap 2016). Palmer amaranth evolved cross-resistance to many ALS-inhibitors. Imazethapyr-resistant Palmer amaranth from KS was found to be approximately 2800 times more resistant compared to sensitive biotype. The same Palmer amaranth population was also resistant to the sulfonylurea herbicides, thifensulfuron and chlorimuron. ALS enzyme inhibition assays support that the resistance is due to an insensitive ALS enzyme, possibly because of mutations in the target-site (Sprague et al., 1997). Similarly, imizaquin-resistant Palmer amaranth from AR was cross-resistant to chlorimuron, diclosulam, and pyrithiobac (Burgos et al., 2001). However, the specific mutation/s contributing to the resistance of imidazolinone/sulfonylurea herbicides is unknown in Palmer amaranth.

EPSPS-Inhibitor resistance

Glyphosate-resistant Palmer amaranth was first reported in 2004 in Georgia (Culpepper et al., 2006) and subsequently started to spread across the US. The Palmer amaranth resistance to glyphosate was found to be due to amplifiction of *EPSPS* gene, the molecular target of glyphosate. Glyphosate-resistant Palmer amaranth has >100 *EPSPS* gene copies, which are distributed all over the genome and functionally correlate to the increase in enzyme expression to resist high rates of glyphosate (Gaines et al., 2010). The low levels of resistance because of reduced uptake and translocation of glyphosate was also reported in Palmer amaranth (Steckel et al., 2008; Nandula et al., 2012).

HPPD-Inhibitor Resistance

Palmer amaranth evolved resistance to HPPD-inhibitors in 2009 and confirmed in Kansas (Thompson et al., 2012) and Nebraska (Sandell et al., 2012). However, the waterhemp is the first weed species that evolved HPPD-inhibitor resistance in 2009. Both waterhemp and Palmer amaranth populations are resistant to all classes of HPPD-inhibitors (e.g. isoxaflutole, mesotrione, tembotrione, and topramezone) with varying levels of resistance to each herbicide (McMullan and Green 2011; Thompson et al., 2012). Enhanced rate of metabolism as mechanism of resistance to mesotrione was documented in waterhemp (Ma et al., 2013). However, the mechanism of resistance in Palmer amaranth is not known yet.

PPO-Inhibitor Resistance

PPO-inhibitors are contact herbicides and inhibit the conversion of protoporphyrinogen IX to protoporhyrin IX, subsequently affecting the biosynthesis of chlorophyll (Deybach et al., 1985). Palmer amaranth resistant to PPO herbicides was first reported in 2011 in a soybean field in Arkansas and later documented in Tennessee in 2015. The mechanism of resistance to

fomesafen in Palmer amaranth was identified as deletion of glycine210 as reported in PPO-resistant waterhemp (Salas et al., 2016; Patzoldt et al., 2006).

Multiple Herbicide Resistance in Palmer Amaranth from KS

The focus of this dissertation is towards understanding the mechanism(s) of multiple herbicide resistance in a population of Palmer amaranth from Kansas. The population was reported to be resistant to herbicides with three different modes of action: PS II-, ALS-, and HPPD-inhibitors (Thompson 2009). A comprehensive review of evolution of resistance to these herbicide groups in various weed species is given below.

Resistance to PS II-Inhibitors

The target-site of chemical groups such as triazine, triazinone, uracil, nitrile, benzothiadiazinone and urea of the PS II-inhibitors is the *psbA* gene in the chloroplast which encodes D1 protein. In the photosynthetic electron transport, D1 protein is an important component that acts as the plastoquinone binding site. PS II-inhibitors competitively bind to the D1 protein replacing the plastoquinone (Gronwald 1994). A wide number of mutations have been identified in photosynthetic bacteria and algae that confer resistance and cross resistance to different classes of PS II-inhibitors (Oettmeier 1999). Triazine resistance in a number of weed species is commonly endowed as a result of substitution of serine264glycine in *psbA* gene (Gronwald 1994). High resolution X-ray crystallographic structures of D1 protein of purple bacteria in complex with plastoquinone and atrazine revealed the specific amino acids of the protein interacting with the plastoquinone and atrazine (Lancaster and Michel 1999). Plastoquinone is hydrogen bonded with amino acids histidine215 and serine264 at the plastoquinone binding site along with other hydrophobic interactions. However, atrazine is

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hydrogen bonded with phenylalanine265 and serine264 preventing the binding of plastoquinone (Fuerst and Norman 1991).

The first case of PS II-inhibitor (triazine) resistance was reported in common groundsel (Ryan 1970), and since then triazine resistance has become most prevalent and documented in many weeds such as common lambsquarters (*Chenopodium album*), wild radish, (*Raphanus raphanistrum*), pigweeds (Bandeen and Mclaren 1976; Foes et al., 1998; Friesen and Powles, 2007). To date 73 weed species resistant to PS II-inhibitors has been confirmed worldwide (Heap 2016).

The point mutations in the target-site, *psbA* gene provides high level of triazine resistance and simultaneously compromises the binding affinity of plastoquinone resulting in reduced photosynthesis (Gronwald 1994; Arntz et al., 2000). In addition, the *psbA* gene is encoded in the chloroplast genome and thus target-site based resistance to triazines is maternally inherited (Holt et al., 1993; Patzoldt et al., 2003). With high fitness cost due to reduced photosynthesis, the triazine-resistant weeds are less competitive compared to the susceptible biotypes and so are present at low frequencies in the weed populations (Jasieniuk et al., 1996). Evolution of herbicide-resistant weeds exhibiting fitness penalty may have positive implications in management of these weeds. For example, in case of evolution of maternally inherited atrazine resistance in weeds associated with fitness penalty, when the atrazine selection is removed, the susceptibles out perform the resistant plants. Thereby, the susceptible plants become dominant and eventually the resistant plants disappear from the population (Jasieniuk et al., 1996). However, this may not be the case with nuclear inherited atrazine resistance, because the herbicide resistance can spread via both pollen and seed. Though the target-site based resistance to atrazine is widespread, non target-site resistance has also been reported in rigid ryegrass,

velevetleaf, blackgrass, through enhanced metabolism of atrazine or simazine via GST or cytochrome P450-mediated detoxification mechanisms (Burnet et al., 1993; Gray et al., 1996; Cummins et al., 1999). Similarly, in waterhemp, a close relative of Palmer amaranth, the non-target site atrazine resistance due to elevated rates of metabolism via GST activity has also been confirmed (Ma et al., 2013).

Non-triazines such as substituted ureas have a different binding site on D1 protein and are effective in managing triazine-resistant weeds, because serine264glycine mutation does not affect the binding of these herbicides to plasoquinone binding site (Arntzen et al., 1982; Trebst, 1991). However, the serine264glycine substitution confers resistant to triazinones, uracils, and pyridazinones (Fuerst et al., 1986). There are five known mutations that confer resistance to non-triazines such as diuron and linuron, hexazinone in few weeds. Valine219isoleucine confer resistance in kochia, annual bluegrass (*Poa annua)* to diuron and Powell amaranth to linuron (Mengistu et al., 2005; 2000; Dumont and Tardif 2002). Alanine251valine substitution is found in Swedish population of common lambsquarters (Mechant et al., 2008), phenylalanine255isoleucine confers resistance to hexazinone in shepherd's-purse (*Capsella bursa-pastoris*) and is susceptible to atrazine, diuron, and terbacil (Perez-Jones et al., 2009). The point mutation asparagines266threonine in a common grounsel population from Oregon conferred resistance to bromoxynil (Park and Mallory-Smith 2006). Common purslane (*Portulaca oleracea*) selected by triazines and ureas with different chemistries has the serine264threonine substitution on the D1 protein (Masabni and Zandstra 1999). The degree of sensitivity of the active site to different classes of PS II-inhibitors in weeds might be due to the differences in the structure of the reaction centre proteins between species.

Resistance to ALS-Inhibitors

ALS is the target-site of many ALS-inhibitors like chlorsulfuron, thifensulfuron, imazamox, propoxycarbazone, pyrithiobac, flumetsulam in different chemical groups. These herbicides inhibit the biosynthesis of branched chain amino acids and are very effective at low doses with soil residual activity, have low mammalian toxicity with broad selectivity in majority of the crops (Ray 1984; Mazur and Falco 1989). All these characteristics resulted in the widespread adoption of ALS-inhibitors. However, with in five years after introduction, rapid evolution of resistance to these herbicides has been reported in a number of weed secies. Some of the first ALS-inhibitor resistant (chlorsulfuron) weeds are prickly lettuce and kochia (Mallory-Smith et al., 1990; Primiani et al., 1990). There are about 97 dicot and 62 monocot weed species resistant to ALS-inhibitors that have been confirmed worldwide to date (Heap 2016). Resistance to ALS-inhibitors ranks the first with highest number of resistant weeds and is posing a serious threat to management and sustainable agriculture. In corn and soybean, the use of ALS-inhibitors is not recommended due to widespread occurrence of resistance in waterhemp populations across the US Midwest. Similarly, resistance to these herbicides is also prevalent in kochia across the US and Canada where wheat is cultivated intensively (Patzoldt et al., 2002; Guttieri et al., 1995).

Target-site based ALS-inhibitor resistance is common in weeds due to point mutations in the *ALS* gene resulting in single amino acid substitutions. Field evolved ALS-inhibitor resistant weeds show substitutions at one of the eight conserved amino acid positions (Powles and Yu 2010). The number of amino acid substitutions dramatically increased as the number of weeds resistant to ALS-inhibitors increased. In 2002, 13 amino acid substitutions across five amino acid positions were documented and increased to a total of 24 amino acid substitutions across eight codons (Tranel et al., 2016). Proline197 residue is the most commonly prone to point mutations

with 11 substitutions reported so far. Kochia populations collected from different locations across United States and Canada showed six amino acid substitutions, threonine, arginine, leucine, glutamine, serine or alanine at proline197 (Guttieri et al., 1995). In addition to these mutations, in certain kochia populations, tryptophan574leucine substitution has also been reported (Foes et al., 1999). Many pigweeds such as waterhemp, redroot pigweed, and Powell amaranth, amino acid substitutions were reported at five of eight positions. Waterhemp is resistant to both imidazolinone (IMI) and sulfonylurea (SU) herbicides of dissimilar chemistries and was found to have tryptophan574leucine, serine653threonine or asparagine substitution (Patzoldt and Tranel 2007). Smooth pigweed with mutations on *ALS* gene at alanine122/205, aspartate376, tryptophan574 or serine653 confers resistance to broad spectrum of ALS-inhibitors (Whaley et al., 2007). Imazethapyr-resistant Palmer amaranth from KS was found to be approximately 2800 times more resistant compared to sensitive biotype and was also shown to be cross resistant to sulfonylurea herbicides, thifensulfuron and chlorimuron (Sprague et al., 1997). However, the precise mechanism of resistance to ALS-inhibitors in Palmer amaranth is not known.

A multiple herbicide resistant waterhemp population from IL showed moderate levels of resistance to ALS-inhibitors. ALS enzyme assays showed that resistance was not due to alteration in the target-site suggesting the presence of non target-site based mechanism of resistance in this weed (Guo et al., 2014). Similarly, metablism-based resistance to ALS-inhibitor has been reported in wild mustard, a common weed in canola and other Brassicaceae crops (Veldhuis et al., 2000). However, this wild mustard population was susceptible to SU and IMI herbicides. Many crops that are naturally tolerant to ALS-inhibitors metabolize these herbicides via activity of cytochrome P450 monooxygenases. Similarly, many grass weeds also evolved

resistance to ALS-inhibitors through non-target-site resistance mechanisms. Resistance in rigid ryegrass selected by ACCase-inhibitors exhibits cross resistance to ALS-inhibitors and the mechanism of resistance was identified as rapid metabolism of chlorsulfuron mediated by P450s (Cotterman et al., 1992). On the other hand, use of malathion, a P450 inhibitor antagonized chlorsulfuron resistance but not resistance to ACCase-inhbitors suggesting that different cytochrome P450's are involved in multiple herbicide resistance in rigid ryegrass (Christopher et al., 1994). Specific cytochrome P450s, *viz.*, CYP71AK2 and CYP72A254 were induced by bispyribac (pyrimidinyltthiobenzoate) treatment in resistant watergrass, a common weed in rice (Iwakami et al., 2014).

Crystal structure and molecular interactions of yeast and *Arabidopsis* ALS in complex with herbicides (chlorsulfuron, sulfometuron methyl and imazaquin) provided a comprehensive understanding of the molecular interactions between the enzyme and herbicide (Fang et al., 2002; McCourt et al., 2005, 2006). ALS has both catalytic and regulatory subunits. The regulatory subunit responds to the substrate or the products (branched-chain amino acids) and activates the catalytic subunit. These studies have revealed the position of catalytic site which is located deep inside a channel in the protein structure showing that herbicides do not bind directly to the catalytic site (Fang et al., 2003; McCourt et al., 2006). Rather, herbicide binds to a domain that is close to the channel entry blocking the substrate access to the active catalytic site. Different ALS-inhibitors orient and interact with amino acid residues on the domain with partial overlapping among the herbicides (McCourt et al., 2006). These structural features explain why certain amino acid substitutions in the herbicide binding domain confer resistance to some and not to other ALS-inhibitors. The high frequency of ALS-inhibitor resistant weeds with wide range of point mutations in a population explains that these alterations prevent the binding of

herbicide in such a way that the access to the substrate is not blocked and produces branched-chain amino acids for growth and development of the weeds (Preston and Powles 2002).

Resistance to HPPD-Inhibitors

HPPD-inhibitors include three chemical classes: isoxazoles, pyrazolones and triketones. HPPD-inhibitors are a relatively new chemicals commercialized to manage wide spectrum of broadleaf and grass weeds. These herbicides competitively inhibits HPPD enzyme and subsequently affects carotenoid biosynthesis in susceptible plants (Beaudegnies et al., 2009). Selectivity and tolerance to HPPD-inhibitors in crops such as corn is primarily due to rapid metabolism, via ring hydroxylation mediated by cytochrome P450s. Also, reduced uptake of mesotrione (HPPD-inhibitors) has been attributed to the selectivity (Mitchell et al., 2001). Soybean is very sensitive to HPPD-inhibitors and recently, transgenic soybeans tolerant to mesotrione, tembotrione and isoxaflutole have been developed (Siehl et al., 2014). So far, only in two weed species, common waterhemp and Palmer amaranth evolution of resistance to HPPD-inhibitors have been documented from IL and KS, respectively (Hausman et al., 2011; Thompson 2012). The mechanism of mesotrione resistance in waterhemp population was shown to be non-target-site based detoxification mediated by cytochrome P450s (Ma et al., 2013).

X-ray crystal structures of maize and *Arabidopsis* HPPD were determined in the absence of their substrate to provide the structural basis for herbicide binding as well as development of new herbicide molecules. The structure reveals that C-terminal helix provides substrate access to the active site (Fritze et al., 2004). However, there are minor differences between the maize and *Arabidopsis* HPPD proteins at the catalytic site.

As discussed above, the mechanisms of resistance to the PS II-, ALS- and HPPD-inhibitors have been well characterized in many weeds. However, the mechanism(s) of resistance

to these three herbicides is not known in Palmer amaranth. The research is based on the hypothesis that the mechanism of resistance in this multiple herbicide resistant Palmer amaranth is due to target-site and/or non-target-site based resistance mechanism. The overall goal of this dissertation was to investigate the physiological, biochemical and molecular basis of multiple herbicide resistance in Palmer amaranth from KS. Mesotrione is used to investigate the mechanism of resistance to HPPD-inhibitors. Similarly, atrazine and chlorsulfuron were used to determine the mechanism of resistance to PS II- and ALS-inhibitors, respectively, relative to known susceptible populations from KS and Mississippi. The specific objectives of this disseration include:

Chapter 2: to: a) determine the level of resistance to mesotrione in Palmer amaranth; b) study the mesotrione absorption and translocation profiles; c) investigate the non-target (metabolism) based resistance to mesotrione; and d) examine the target-site alterations in *HPPD* gene by sequencing or quantify the *HPPD* gene expression.

Chapter 3: to: a) determine the level of resistance to atrazine in the same Palmer amaranth; b) examine the target-site alterations in *psbA* gene by sequencing; c) investigate the GST-based conjugation of atrazine; and d) examine nuclear or maternal inheritance of atrazine resistance.

Chapter 4: to a) determine the level of resistance to chlorsulfuron; b) examine the target-site mutations in *ALS* gene by sequencing; and c) evaluate the cross resistance to other chemical classes of ALS-inhibitors.

Understanding the mechanism of multiple herbicide resistance in Palmer amaranth provides an insight on how weeds withstand the lethal effects of herbicides and helps in designing alternate weed management strategies. Effective management of herbicide resistant weeds is very important to minimize the evolution and spread of resistance. The most common

strategy is to reduce the intensity of herbicide selection by sequential application of herbicide mixtures or rotate with herbicides with different modes of action over multiple growing seasons (Delye et al., 2013). This helps in reducing the probability of survival and reproduction of resistant individuals in the population. Management strategies that reduce/eliminate the spread of resistance by means of pollen movement, seed production, and propagule dispersal and the additions of resistant weed seed to the soil seed bank are very effective. However, it is not possible without the integrated management practices such as planting weed free crop, utilizing crop competitiveness, rotating crops, appropriate timing and tillage (Norsworthy et al., 2012). Evolution of resistance in weeds and their spread cannot be prevented as long as herbicides are used but can be delayed by understanding the genetic basis and mechanism of herbicide resistance, as well as following integrated weed management strategies.

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Chapter 2 - Rapid Detoxification Combined with Increased Expression of HydroxyPhenylPyruvate Dioxygenase (HPPD) Confer Resistance to Mesotrione in Palmer Amaranth (*Amaranthus palmeri*)

Abstract

Herbicides that inhibit hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors (e.g. mesotrione) are widely used to control a broad spectrum of weeds in agriculture. *Amaranthus palmeri* is an economically troublesome weed throughout the US. The first case of evolution of resistance to HPPD-inhibiting herbicides in *A. palmeri* was documented in KS and later NE. The objective of this study was to characterize the biochemical and molecular basis of mesotrione resistance in Palmer amaranth. Investigation of HPPD-resistant *A. palmeri* that was previously known to be multiple herbicide resistant, revealed that these populations are 10-18 times more resistant than their sensitive counterparts. Analysis of absorbtion and translocation of [^{14}C] labeled mesotrione suggested no difference that can explain the resistance. Importantly, mesotrione (>90%) was detoxified markedly faster in the resistant populations, within 24 hours after treatment (HAT) compared to sensitive plants. However, at 48 HAT all populations metabolized the mesotrione, suggesting additional factors may contribute to this resistance. Further evaluation of HPPD-resistant *A. palmeri* did not reveal any specific resistance-conferring mutations nor amplification of *HPPD* gene, the molecular target of mesotrione, however, the HPPD-resistant population showed 4-12 fold increased levels of the *HPPD* transcript. This increase in *HPPD* transcript levels was accompanied by increased HPPD protein expression. The significant aspects of this research include contribution of both non-target-site based (rapid detoxification) and target-site based (increased gene expression) mechanisms in the evolution of herbicide resistance in a naturally occurring weed species.

Introduction

Mesotrione (Callisto[TM]) is a synthetic triketone herbicide chemically known as 2-[4-(methylsulfonyl)-2-nitrobenzoyl]- 1, 3-cyclohexanedione and biochemically inhibits 4-hydroxyphenylpyruvate dioxygenase (HPPD). HPPD is a key enzyme in the catabolism of tyrosine and anabolism of plastoquinones, tocopherols, and subsequently carotenoid biosynthesis (Beaudegnies et al., 2009). Plastoquinone plays a vital role in two significant pathways: a) as an essential component of photosynthetic electron transfer from photosystem II to photosystem I in the process of generating ATP and b) acts as an important cofactor for phytoene desaturase, a key enzyme in the carotenoid biosynthesis pathway. Carotenoids are light harvesting molecules and protect plants from photo oxidation by quenching the triplet chlorophyll and prevent the formation of destructive singlet oxygen (Siefermann 1987).

HPPD-inhibitors are a relatively new class of chemistry discovered about three decades ago and are widely used in agriculture for weed management. HPPD-inhibitors are broadly classified into three chemical families: Isoxazoles (e.g. isoxaflutole and pyrasulfotole), Pyrazolones (e.g. topramezone), and Triketones (e.g. mesotrione and tembotrione) depending on the chemical structure and properties. Upon treatment with these herbicides, susceptible plants exhibit characteristic bleaching symptoms as a result of destruction of carotenoids and eventually leading to lipid peroxidation of cell membranes. Mesotrione is one of the most widely used HPPD-inhibiting herbicides that selectively control many broad-leaved weeds, including Palmer amaranth, and some grasses in corn agriculture when applied post as well as pre emergence (Mitchell et al., 2001). Rapid metabolism, via ring hydroxylation mediated by cytochrome P450 monooxygenase(s) combined with reduced uptake of mesotrione has been attributed to selectivity of this herbicide in corn (Mitchell et al., 2001). On the other hand, the differential

selectivity of mesotrione between monocot and dicot species is attributed to HPPD enzyme in monocots being less sensitive to the inhibitors. Tobacco, a dicot species is highly sensitive to mesotrione, however, when transformed with a *HPPD* gene from wheat, showed tolerance to this herbicide (Hawkes et al., 2001). Transgenic soybeans tolerant to mesotrione, tembotrione and isoxaflutole have been developed to increase the selectivity and spectrum of weed control (Siehl et al., 2014). The significance of mesotrione and other HPPD-inhibitors has been reported in controlling several acetolactate synthase- and photosystem II-inhibitor resistant weed biotypes (Sutton et al., 2002). It is also important to preserve the effectiveness and extend the use of these herbicides as no new herbicides have been introduced in the last 20 years and new herbicide resistant traits are being stacked in crops to control weeds.

Palmer amaranth (*Amaranthus palmeri*) is one of the most economically important weeds in corn (*Zea mays* L.), soybean (*Glycine max* L.), cotton (*Gossypium* spp.), sorghum (*Sorghum bicolor* L.) and many other cropping systems throughout the US. Infestation of Palmer amaranth can significantly decrease the quality and cause huge yield losses ranging from 63-91% depending on the density and duration of interference in different crops (Ward et al., 2013). Management of Palmer amaranth is possible using several herbicide chemistries, however, repeated and extensive use of herbicides resulted in the evolution of resistance to multiple herbicides with various modes of action such as 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, acetolactate synthase (ALS)-, photosystem II (PS II)-, microtubule-, more recently to protoporphyrinogen oxidase (PPO)- and HPPD-inhibitor herbicides (Heap, 2015). Currently, two weed species in the Amaranthaceae family, common waterhemp (*Amaranthus tuberculatus*) and Palmer amaranth, have evolved resistance to several HPPD-inhibiting herbicides which offer a feasible option to manage other herbicide-resistant weeds including glyphosate-resistant Palmer

amaranth (Norsworthy et al., 2008). The HPPD-inhibitor resistant waterhemp was first reported in IL in 2009 (Hausman et al., 2011). Detoxification possibly mediated by cytochrome P450 monooxygenases has been attributed to confer mesotrione resistance in this waterhemp population (Ma et al., 2013).

In central KS, a Palmer amaranth population with resistance to HPPD-inhibitors was first documented in Stafford County and subsequently confirmed in 2012 (Thompson 2009; 2012). Later, HPPD-inhibitor resistant Palmer amaranth populations were also found in the nearby state of NE in a cornfield, which had a history of continuous use of HPPD-inhibitors (Sandell et al., 2012). Interestingly, the field in KS where HPPD-inhibitor-resistant Palmer amaranth was found, had no previous history of applications of HPPD-inhibitors, but did have a long history of PS II- and ALS-inhibiting herbicides. This was initially found resistant to Huskie®, a premix of pyrasulfotole (HPPD-inhibitor) and bromoxynil (PS II-inhibitor) and is also resistance to several other HPPD-inhibitors such as mesotrione, tembotrione, and topramezone and was also found to be resistant to atrazine, a widely used PS-II inhibitor (Thompson et al., 2010, 2012). The mechanism of HPPD-inhibitor resistance in the Palmer amaranth populations from KS or NE is unknown. The research is based on the hypothesis that HPPD-inhibitor resistance in Palmer amaranth is mediated either by a target-site or a non-target-site resistance mechanism. The objectives of this research were to determine the mechanism(s) of resistance to mesotrione in the HPPD-inhibitor resistant Palmer amaranth populations from KS and NE.

Materials and Methods

Plant Material and Growth Conditions

Three mesotrione resistant Palmer amaranth populations from KS and NE, designated as KSR, KSR2, NER and three mesotrione sensitive populations from MS, KS and NE, designated

as MSS, KSS and NES, respectively were used in this study. KSR seed was derived by crossing male and female plants of Palmer amaranth from KSR2 that survived 105 g ai ha^{-1} (field use rate of mesotrione) under greenhouse conditions to generate a more homogeneous resistant population. However, KSR2 seed was collected from Palmer amaranth plants that survived a HPPD-inhibitor application in a field in Stafford County, KS (Thompson et al., 2012) that had wheat-sorghum crop rotation. Seed of NER was collected from Palmer amaranth that survived mesotrione application in a cornfield in NE (Sandell et al., 2012). Seeds of mesotrione-susceptible and -resistant Palmer amaranth were germinated in small trays (25 x 15 x 2.5 cm) with commercial potting mixture (Miracle Gro). Seedlings 2-3 cm tall, were transplanted into small pots (6 x 6 x 6.5 cm) in the greenhouse, maintained at 25/20 °C day/night and 15/9 h photoperiod, supplemented with 250 µmol m^{-2} s^{-1} illumination provided with sodium vapor lamps. When the plants reached 5-6 cm tall, they were transferred to a growth chambers maintained at 32.5/22.5 °C day/night, 15/9 h photoperiod, 60-70% relative humidity. Light in the growth chamber was provided by fluorescent bulbs delivering 550 µmol m^{-2} s^{-1} photon flux density at plant canopy level. Plants were watered as needed regularly both under greenhouse as well as growth chamber conditions.

Mesotrione Dose Response Assay

Mesotrione-resistant (KSR) and -susceptible (MSS and KSS) Palmer amaranth were grown under greenhouse and growth chamber conditions as described above. Initially, the KSR and KSR2 Palmer amaranth populations were screened with the commercial field application rate of 105 g ai ha^{-1} mesotrione (Callisto[TM], Syngenta) to determine the frequency of resistant individuals in the population before determining the level of resistance by dose response assay. The frequency of resistance was 90-95% and 60-70% in KSR and KSR2, respectively (data not

shown). For the dose response analysis, when the Palmer amaranth plants (MSS, KSS and KSR) were 10-12 cm tall with 8-10 leaves, mesotrione was applied at 0, 6.5, 13.125, 26.25, 52.5, 105 (1X), 210, 315, 420 and 840 g ai h^{-1}, where 1X represents the field recommended rate of mesotrione. Required adjuvants, crop oil concentrate (COC, Agridex) and ammonium sulfate (AMS, Liquid N-Pak; Winfield) at 1% v/v and 2.5% w/v (8.5 lb/100 gal), were included, respectively in all the treatments. Treatments were applied with a bench-type track sprayer (Generation III, De Vries Manufacturing, RR 1 Box 184, Hollandale, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL) delivering 187 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. Following treatment, plants were returned to the same growth chambers (within 30 min after treatment). Treatments were arranged in a completely randomized design with five replications and the experiment was repeated three times. Treated plants were clipped off at the soil surface and immediately weighed (aboveground fresh biomass) 3 weeks after treatment (WAT). Harvested plants were packed in paper bags and oven dried at 60°C for a week before weighing dry biomass.

Absorption and Translocation of [^{14}C] Mesotrione

Greenhouse grown seedlings (as described above) of KSR and MSS and KSS Palmer amaranth were moved to growth chamber 2-3 days before applying [^{14}C] mesotrione to allow the plants to acclimate. Ten to 12 cm tall (8-10 leaf stage) plants were treated with a total of 3.3 k Bq of [phenyl-U-^{14}C]-labeled mesotrione with specific activity of 781 M Bq g^{-1}. Unlabeled mesotrione was added to the radioactive solution to obtain 105 g ai h^{-1} mesotrione in a carrier volume of 187 L. Additionally, crop oil concentrate (COC, Agridex) and ammonium sulfate (AMS, Liquid N-Pak; Winfield) were added at 1% v/v and 1% w/v, respectively to this mixture to enhance droplet-to-leaf surface contact. A total volume of 10 µL was applied as ten 1 µL

droplets on the upper surface of the fourth youngest leaf. The treated plants were returned to the same growth chamber. Plants were harvested at 48 and 72 hours after treatment (HAT) and separated into treated leaf (TL), leaves above the treated leaf (ATL), and leaves below the treated leaf (BTL) and wrapped in a single layer of tissue paper. Treated leaves were washed with 5 mL wash solution (10% methanol and 0.05% Tween) for 60 seconds in a 20 mL scintillation vial to remove any unabsorbed herbicide. Radioactivity in the leaf rinsate was measured using liquid scintillation spectrometry (LSS: Tricarb 2100 TR Liquid Scintillation Analyzer; Packard Instrument Co., Meriden, CT). Plant parts were oven dried at 60°C for 48 h and total radioactivity absorbed was quantified by combusting using a biological oxidizer (OX-501, RJ Harvey Instrument) and LSS. Total mesotrione absorption was determined as; % absorption = (total radioactivity applied – radioactivity recovered in wash solution) x 100 / total radioactivity applied. Herbicide translocation was determined as; % translocation = 100 – % radioactivity recovered in treated leaf, where % radioactivity recovered in treated leaf = radioactivity recovered in treated leaf x 100 / radioactivity absorbed. Six replications were included in each treatment and the experiment was repeated.

Metabolism of Mesotrione in Whole Plant and Treated Leaves

KSR, NER and MSS, KSS and NES Palmer amaranth populations were grown as described previously for [14C] mesotrione absorption and translocation experiments. Twenty µL of [14C] mesotrione containing 7.2 k Bq was applied on 10 to 12 cm tall (8 to 10 leaf stage) plants as ten 1µL droplets on the adaxial surface of fully expanded fourth and fifth youngest leaves. [14C] mesotrione and its metabolites were extracted as described in Godar et al., 2015. Treated leaves were harvested 4, 8, 16, 24, 48 and 72 HAT and washed with wash solution to remove unabsorbed herbicide. Whole plant tissue including the washed treated leaves or only the

treated leaves were then frozen in liquid nitrogen and homogenized using a mortar and pestle. [^{14}C] mesotrione and its metabolites were extracted with 15 ml of 90% acetone at 4°C for 16 h. The samples were centrifuged at 6500 rpm (5,000 g) for 10 min and supernatant from each sample was concentrated at 45°C for 2-3 h with a rotary evaporator (Centrivap, Labconoco) until a final volume of 500-1000 µL of extract was reached. The extract was then transferred to a 1.5 mL microcentrifuge tube and centrifuged at high speed (13,000 rpm/10,000 g) for 10 min at room temperature. The total radioactivity in each sample was measured by LSS and samples were normalized to 0.05 KBq/50 µL (3000 dpm/50µL) amount of [^{14}C]-labeled compounds by diluting the samples with acetonitrile:water (50:50, v/v) prior to HPLC analysis.

Total extractable radioactivity in 50 µL was resolved into parent [^{14}C] mesotrione and its polar metabolites by reverse-phase HPLC (Beckman Coulter, System Gold) following the protocol optimized previously in our laboratory (Godar et al., 2015). Reverse-phase HPLC was performed with a Zorbax SB-C18 column (4.6 x 250 mm, 5-µm particle size; Agilent Technologies) at a flow rate of 1 mL min^{-1}. The radioactivity in the sample was detected using radio flow detector LB 5009 (Berthold Technologies). The whole plant metabolism experiment had three replicates for each treatment and the experiment was repeated. Similarly, the experiment where metabolism of mesotrione in only treated leaf was performed also included three replicates and was repeated.

RNA Extraction, cDNA Synthesis, and *HPPD* Gene Expression

In this study, the KSR, NER and MSS, KSS and NES Palmer amaranth plants were not treated with mesotrione, however, adjuvants COC (1% v/v) and AMS (0.85% w/v) were applied to 10-12 cm tall plants. Above ground plant tissue was harvested 24 h after treatment and frozen in liquid nitrogen and stored at –80°C for RNA isolation. The frozen tissue was homogenized in

liquid nitrogen using a pre-chilled mortar and pestle to prevent thawing and transferred 100 mg

tissue into a 1.5 mL microcentrifuge tube. Total RNA was isolated using RNeasy Plant Mini Kit

(Qiagen Inc., Valencia, CA, USA). The quality and quantity of total RNA was determined using

agarose gel (1%) electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific)

and RNA was stored at -80°C.

For cDNA synthesis, 1 µg of total RNA was treated with DNase I enzyme (Thermo

Scientific, Waltham, MA, USA) to remove any genomic DNA. cDNA was synthesized from 1

µg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and was

diluted in 1:5 ratio for gene expression study. Quantitative PCR/real time PCR (qPCR/rtPCR)

was used to determine *HPPD* gene expression in all samples. The qPCR reaction mix consisted

of 8 µL of SYBR Green mastermix (BioRad Inc., Hercules, CA, USA), 2 µL each of forward

and reverse primers (5 µM), and 20 ng cDNA to make the total reaction volume of 14 µL. *HPPD*

gene expression was normalized using either β-tubulin or carbamoyl phosphate synthetase (*CPS*)

as a reference gene. qPCR (CFX96 Touch™ Real-Time PCR Detection System, BioRad Inc.)

was performed at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s and 60°C for

1 min (Ma et al., 2013). A meltcurve profile was included following the thermal cycling protocol

to determine the specificity (no primer dimers, no genomic DNA contamination, and no non-

specific product) of the qPCR reaction. Primer sequences used were: HPPD forward and reverse

(F 5'-CTGTCGAAGTAGAAGACGCAG-3'and R 5'-TACATACCGAAGCACAACATCC-3');

β-tubulin forward and reverse (F 5'-ATGTGGGATGCCAAGAACATGATGTG-3' and R 5'-

TCCACTCCACAAAGTAGGAAGAGTTCT-3'); and CPS forward and reverse (F 5'-

ATTGATGCTGCCGAGGATAG-3' and R 5'- GATGCCTCCCTTAGGTTGTTC-3'). The

HPPD:*β*-tubulin and *HPPD:CPS* expression was determined using the $2^{\Delta CT}$ method, where CT is

threshold cycle and ΔCT is $CT_{Reference\ gene\ (\beta\text{-}tubulin,\ or\ CPS)}$ - $CT_{Target\ gene\ (HPPD)}$. HPPD gene expression was studied using three biological replicates and three technical replicates for each biological replicate. The experiment was repeated three times and the average value ± standard error of total biological replicates was used to show the expression fold.

Protein Extraction, SDS-PAGE, and Western Blotting

Above ground plant tissue (0.5 g) from 10-12 cm tall Palmer amaranth from KSR, NER and MSS, KSS and NES was homogenized in liquid nitrogen and added to 20 mL extraction buffer (50 mM Tris-HCl, pH 8 , 50 mM NaCl, 1mM EDTA, 1mM MgCl2 and 0.038 g PMSF, one tablet of Pierce Protease Inhibitor (Thermoscientific), 1g insoluble PVPP). The extraction and purification procedure was developed by modifying the methods of Wang et al., 2006 and Wu et al., 2014. In short, homogenates were centrifuged at 4°C, 10 min, 16000 rpm (Beckman J2-HC centrifuge, USA) and supernatant was collected. One ml of TCA (100%) was added to 10 ml of supernatant and incubated for 1 hr at 4°C. Samples were centrifuged as before, and the supernatant was discarded. Two ml of methanol (100%) was added to the pellet, tubes were vortexed vigorously for 60 seconds and centrifuged (4°C, 10 min, 16000 rpm). Supernatant was discarded and acetone (2 ml; 80%) was added to the pellet, vortexed and then centrifuged (4°C, 10 min, 16000 rpm). Pellet was air dried to remove the remaining acetone and 2 ml phenol (equilibrated with Tris-HCL; pH 8.0, Sigma) was added, vortexed at high speed for 30-60s and centrifuged (4°C, 10 min, 16000 rpm) and the supernatant was collected. Proteins were precipitated by adding 2 mL ammonium acetate (0.1 M in methanol) to the supernatant and incubated overnight at -20°C. Next, the sample was centrifuged (4°C, 10 min, 16000 rpm) and the supernatant was discarded. Pellet was washed with methanol (100%) followed by acetone (80%) and finally air dried. Dried samples were resuspended in 200 µL SDS-ample buffer and

the protein concentration in the extract was determined using the RED 660^{TM} Protein Assay (G-Biosciences).

To resolve proteins in the samples by SDS gel electrophoresis, samples were incubated at 95 °C for 5 minutes. Next, 100 µg of total protein was resolved by electrophoresis on an 11% polyacrylamide gel (90 min at 120V) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) at 150V for 1 hr or 30V overnight. The PVDF membrane was blocked with 5% non-fat dry milk at room temperature for 30 min and then washed three times in tris-buffered salinr with tween 20 (TBST) buffer. The membranes were incubated with a rabbit polyclonal HPD antibody (Novus biologicals; dilution 1:500) in TBST at 4 °C overnight. The membrane was washed three times with TBST and incubated with donkey anti-rabbit HRP conjugated polyclonal antibody (Jackson Immuno Research Laboratories Inc; dilution 1:50,000) at room temperature for 1 hr. After three more washes, membranes were exposed to an HRP substrate solution (Luminata^{TM}, Millipore) and image detection and quantification was carried out using a G-BOX (Syngene).

DNA Extraction and *HPPD* Gene Amplification

DNA extraction for *HPPD* gene amplification was performed on the same plant samples used for RNA extraction, cDNA and *HPPD* gene expression. Genomic DNA (gDNA) was extracted from the frozen leaf tissue (100 mg) using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The quality and quantity of gDNA was determined using agarose gel (0.8%) electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific) and DNA was stored at -20 or -80°C. The following forward and reverse primers (F 5'-CTGTCGAAGTAGAAGACGCAG-3'and R 5'-TACATACCGAAGCACAACATCC-3') were used to amplify the *HPPD* gene from Palmer amaranth populations.

Statistical Analysis

All the experiments were conducted in a completely randomized design and the data from all experiments were combined for each study before performing statistical analysis as there was no interaction between the experiments and treatments.

Dose-response data (expressed as percentage of the untreated control) were analyzed using 'drc' package in R 3.1.2 (Knezevic et al., 2007; R Core Team 2015; Ritz and Streibig 2014; Seefeldt et al., 1995). The three parameter log-logistic model as shown below was used to show the relationship between herbicide rate and biomass,

$Y = d/[1+\exp\{b[\log(x)-\log(GR_{50})]\}]$ where Y is the response (dry biomass or plant health) expressed as percentage of the untreated control, d is asymptotic value of Y at upper limit, b is the slope of the curve around GR_{50} (the herbicide rate giving response halfway between d and the lower asymptotic limit which was set to 0), and x is the herbicide rate. Resistance index (R/S) was calculated as GR_{50} ratio between the MSS or KSS and the KSR populations.

Uptake and translocation data, expressed as percentage of applied and absorbed, respectively, metabolism data, qPCR (*HPPD* gene expression) data were analyzed using one-way ANOVA in R 3.1.2 and the means were compared using Tukey's HSD test. The time course of mesotrione metabolism by MSS and KSR Palmer amaranth populations was fitted with a three-parameter Weibull regression.

Results

Mesotrione Dose Response Assay to Determine the Level of Resistance

The HPPD-inhibitor-resistant and -susceptible Palmer amaranth populations were derived from different locations. To determine their level of resistance to mesotrione in a laboratory setting we conducted dose response assays with these populations. We found a variation in the

level of resistance to mesotrione at individual plant level in all populations, especially the KSR2 (Fig. 2.1 A). This variation is a reflective of genetic variability within and among the populations because the experiments were conducted under controlled environmental conditions (growth chambers) eliminating changes in environmental conditions. Since KSR2 showed extreme variation at 105 g ai ha^{-1} mesotrione, the population was not used further in the dose response analysis. The amount of mesotrione required to reduce plant growth to 50% (GR$_{50}$) 3 weeks after treatment (WAT) was ~151 g ai ha^{-1} for KSR compared to 15 and 8 g ai ha^{-1} for MSS and KSS, respectively (Fig. 2.1 B). However, all the surviving resistant individuals showed injury (bleached) symptoms on shoot meristem at all doses of mesotrione and 3 WAT the injured plants did not recover to phenotype of untreated plants even at low doses of 52.5 g ai ha^{-1} mesotrione. The KSR was 10 and 18 times more resistant compared to MSS and KSS, respectively (Fig. 2.1 B, Table 2.1). In a different study, the NER Palmer amaranth showed 4-14 fold resistance relative to NES in response to mesotrione, tembotrione and topramezone applications (Sandell et al., 2012).
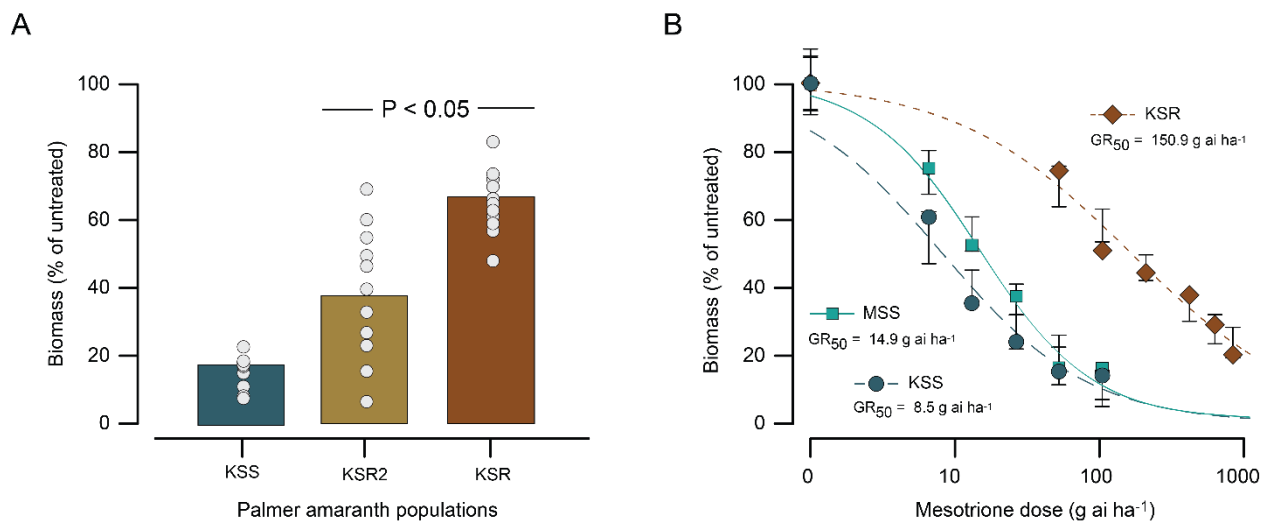
Figure 2.1 Response of susceptible (MSS and KSS) and resistant (KSR) Palmer amaranth populations three weeks after treatment with the herbicide mesotrione. (A) Individual plant aboveground dry biomass variability in response of KSR compared to the original KSR2

population to 105 g ai ha^{-1}rate of mesotrione, bars indicates the average from 12 individual samples (circles). (B) Non-linear regression analysis of aboveground dry biomass of MSS, KSS and KSR populations at different doses of mesotrione. Symbols are averages of twelve replicates fitted with a three parameter log-logistic model; model parameters are shown in Table 2.1.

Uptake and Translocation of [^{14}C] Mesotrione

The resistance/higher tolerance to mesotrione and other HPPD-inhibiting herbicides can arise through a variety of mechanisms. First, we tested if there is a difference in absorption by measuring how much [^{14}C] mesotrione was absorbed by the resistant and susceptible plants. Absorption of [^{14}C] mesotrione in KSR at 48 and 72 hours after treatment (HAT) was 71% and 69% (as % of total applied), which was not significantly different from the susceptible populations (76% and 74% in MSS and 69% and 77% in KSS, at 48 and 72 HAT, respectively; Fig. 2.2 A & B, P > 0.05).

Once absorbed, these herbicides generally translocate via both xylem and phloem (Mitchell et al., 2001; Beaudegnies et al., 2009) to other parts of the plant. Resistance can be derived if the plants have reduced translocation of the herbicide. However, there was no significant difference in the translocation of [^{14}C] mesotrione at 48 HAT between resistant or susceptible populations. Actually, the KSR (37% expressed as % of total [^{14}C] mesotrione absorbed) showed translocation that was in between both susceptible populations MSS (29%) and KSS (55%) populations (Fig. 2.2C, P > 0.05). This suggests there is an underlying genetic variation in the ability of Palmer amaranth to translocate mesotrione that does not correlate with resistance. This variation is likely responsible for the significant difference we observed in the translocation of [^{14}C] mesotrione between the MSS and KSS. Furthermore, the significant difference disappeared at 72 HAT where the KSR, MSS and KSS had 39, 33 and 39%, respectively, of [^{14}C] mesotrione translocated from the treated leaf, to the above and below

treated plant parts (Fig. 2.2 D, P>0.05). Thus, neither difference in mesotrione absorption nor

translocation contributed substantially to the resistance to mesotrione in KSR Palmer amaranth.
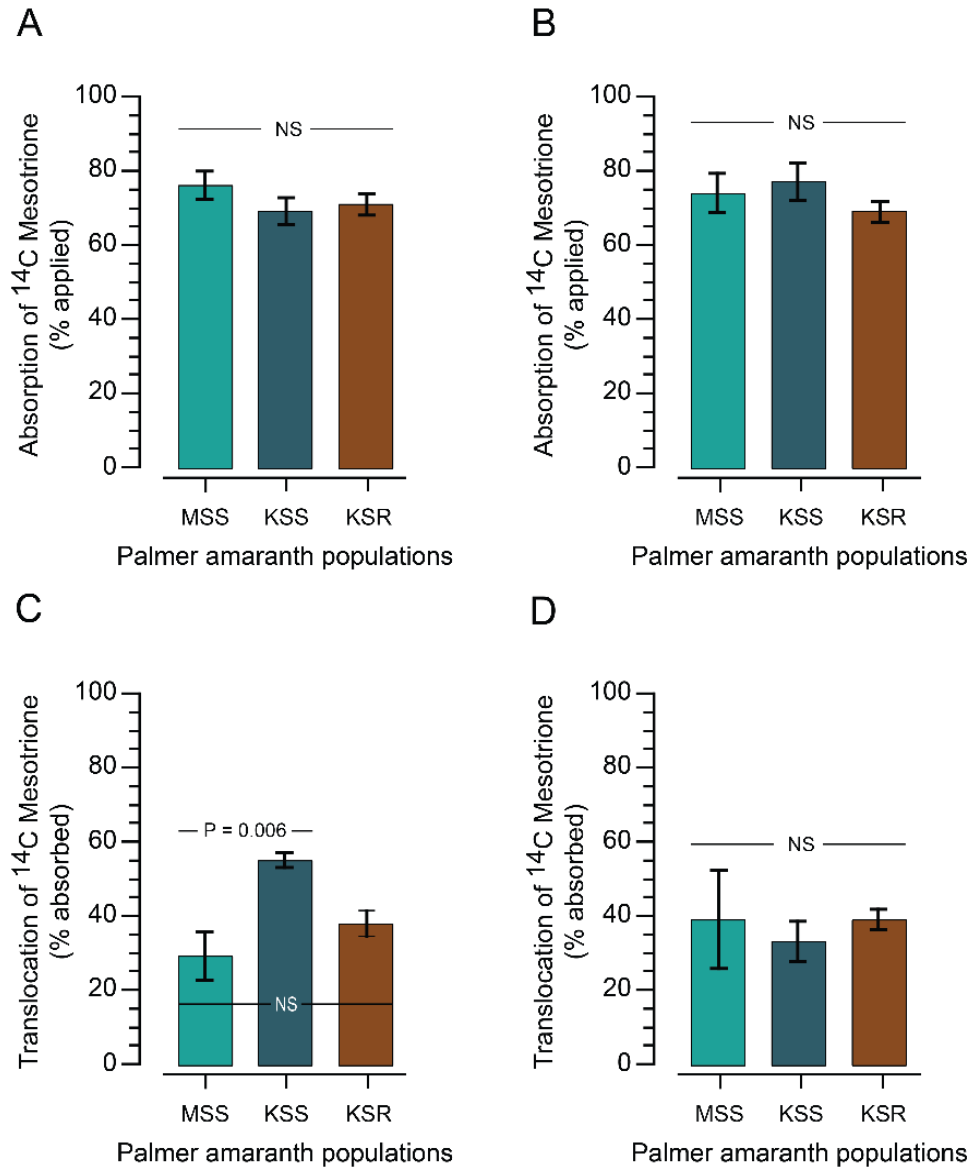
Figure 2.2 (A and B) Absorption and (C and D) translocation of [^{14}C] mesotrione in resistant (KSR) and susceptible (MSS and KSS) Palmer amaranth populations. Uptake and translocation in the plant was measured 48 (A and C) and 72 (B and D) hours after treatment with LSS. Error bars represent standard error of means of 6 biological replicates at each time point. NS = non-significant at P = 0.05.

Metabolism of [^{14}C] Mesotrione

The differential selectivity of mesotrione and many herbicides such as sulfonylureas

(ALS-inhibitors) and triazines (PS II-inhibitors) between crops and weeds is attributed to the

ability of the crops to rapidly detoxify these compounds by cytochrome P450 monooxygenases

or glutathione S-transferases (GSTs) (Hawkes et al., 2001). Some weeds also have been shown

to be able to acquire resistance by increasing their ability to metabolize specific herbicides. To

test for a role of metabolism based resistance in the KSR population, we measured how much

[^{14}C] mesotrione was metabolized into other polar compounds over time. The input [^{14}C]

mesotrione resolved at peak retention time of about 18.1 by reversed-phase HPLC with no other

peaks observed (data not shown). This indicates that peaks at 13.1 and 14.3 retention times

observed in plant lysates are products derived from mesotrione metabolism (Fig. 2.3). These

peaks gradually increased with decrease in input [^{14}C] mesotrione in all the populations

indicating that the metabolites might be hydroxylated products of mesotrione (Ma et al., 2013).

To determine the % of mesotrione remaining, we quantified the amount of radioactivity of the

18.1 peak as fraction of total radioactivity. As early as 4 HAT we observed significant

differences with more than 70% of input parent [^{14}C] mesotrione still being detected in

susceptible samples, while in KSR plants ~50% of parent [^{14}C] mesotrione was metabolized

(data not shown). At 24 HAT, KSR and NER metabolized much more parent compound ($> 90\%$)

compared to MSS, KSS and NES (Fig 2.3 A, B, C, D & E) (P < 0.01), which still showed about

28%, 30% and 50%, respectively, of parent [^{14}C] mesotrione. This amount of mesotrione was

sufficient to injure the plant and subsequently kill the susceptible plants 3 WAT. The half-life T_{50}

is the amount of time taken for 50 % of the parent input [^{14}C] mesotrione to degrade or

metabolize inside the plant through enzymatic transformation. It was found that T_{50} for MSS and

KSR was 5.9 and 14.6 h, respectively, indicating that KSR metabolizes the mesotrione 2.5 times faster compared to the MSS (Supplemental Fig. 2.6 and Table 2.2). Interestingly, both resistant and susceptible Palmer amaranth populations were able to completely metabolize parent [^{14}C] mesotrione by 48-72 HAT (Appendix *A*, Fig. 2.5; 2.6) suggesting that rapid metabolism alone may not be conferring resistance to mesotrione in KSR or NER. The chromatograms of [^{14}C] mesotrione metabolism in MSS, KSS and KSR at 4, 8, 26, 24, 48, and 72 HAT are shown in Appendix A Fig. 2.1-2.6),
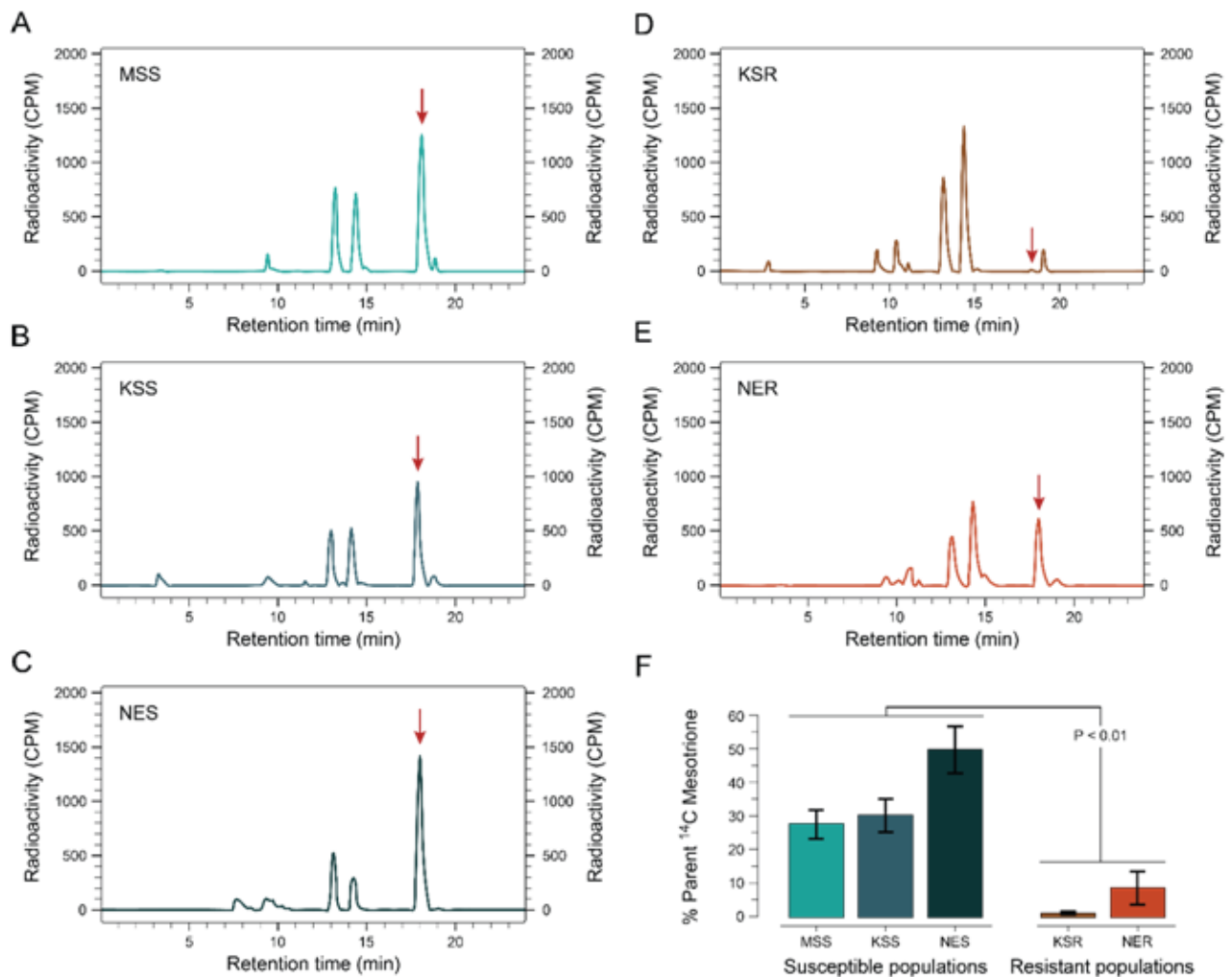
Figure 2.3 Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 24 hours after treatment. Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested (A) MSS (B) KSS (C) NES (D) KSR and (E) NER.

Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione. (F) Represents the amount of [^{14}C] mesotrione input remaining as percentage of total in the resistant (KSR and NER) and susceptible populations (MSS, KSS and NES) 24 HAT. Error bars represent the standard error of means of 6-9 biological replicates.

Analysis of *HPPD* Gene Expression

Biochemically, mesotrione and other HPPD-inhibiting herbicides act as competitive inhibitors of the HPPD enzyme involved in the conversion of 4-hydroxyphenylpyruvate (HPP) to 2, 5-dihydroxyphenylacetate (homogentisate). We hypothesized that, in addition to rapid metabolism, increased expression of the *HPPD* gene may possibly contribute to mesotrione resistance in KSR or NER. To test this idea, mRNA levels of *HPPD* in all mesotrione-resistant and susceptible Palmer amaranth individuals were determined. Since genetic variation exists, there was 1 to 2.5 fold variation in *HPPD* gene expression among the susceptible populations (MSS, KSS and NES). *HPPD* mRNA levels in KSR and NER (normalized against *β-tubulin* and *CPS)* was at least 12 and 8 to12 fold higher, respectively, compared to MSS (Fig. 2.4 A, $P <$ 0.001). When compared to the other two susceptible populations, KSS and NES, *HPPD* gene expression relative to *β-tubulin* or *CPS* was least 4 to 9 fold more in KSR and NER (Fig. 2.4 B and C) ($P = 0.001$). These data indicate that the basal mRNA levels for HPPD are strongly upregulated, irrespective of the exposure to mesotrione.
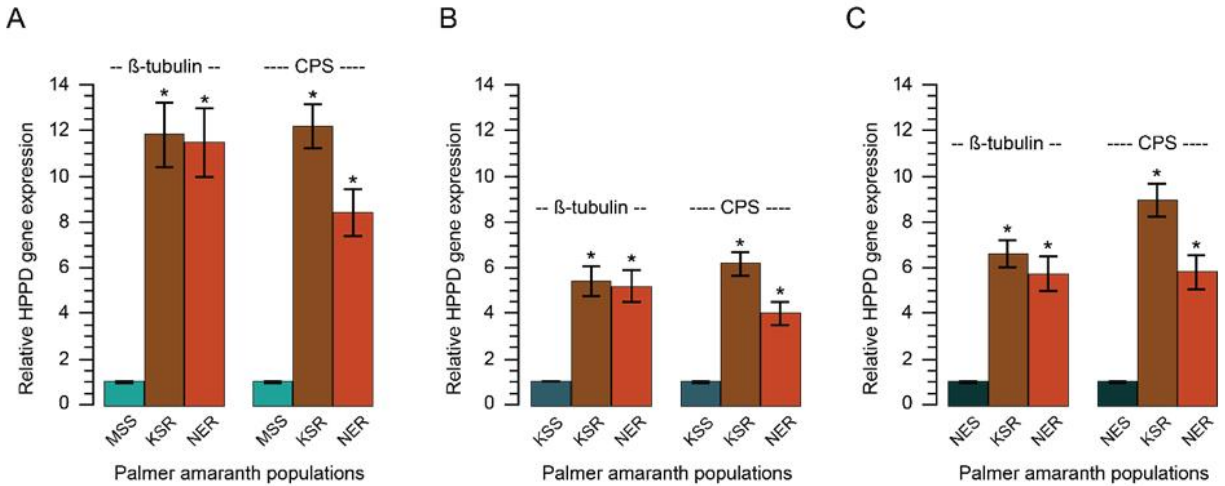
Figure 2.4 The amount of *HPPD* gene expression was normalized to the corresponding level of *β-tubulin* and *CPS*. The amount of *HPPD* gene expression in the susceptible Palmer amaranth populations was normalized to 1, so that the values of resistant Palmer amaranth populations are presented as the fold-increase relative to each susceptible population. *HPPD* gene expression in KSR and NER relative to (A) MSS (B) KSS and (C) NES. Bars represent the means ± SE. Asterisks above error bars represent significant difference in *HPPD* gene expression compared to corresponding reference sample MSS, KSS or NES at α = 0.05. The *HPPD* gene expression 24 HAT with meotrione was similar to untreated (UT) and is shown in Appendix A, Fig. 2.7.

HPPD Protein Expression in Mesotrione-Resistant Palmer Amaranth

To investigate whether the *HPPD* mRNA transcript abundance correlates with increased HPPD protein levels, we next conducted immunoblot analysis. No antibody is available against Palmer amaranth HPPD; however HPPD is 35 % identical with human HPPD. Therefore we used a human HPPD antibody to test if there is cross reactivity with the Palmer amaranth *HPPD* protein. As shown in Figure 5, the antibody recognized HPPD in human cell lysates (HEK lysate). In the Palmer amaranth lysate, a protein with molecular weight of about 48 kDa was detected, which is consistent with the anticipated size of *Amaranthus* HPPD. The protein could be detected in both susceptible and resistant Palmer amaranth populations, however, KSR or

NER lysates showed consistently more HPPD protein as compared to MSS, KSS or NES lysates (Fig. 2.5). The differences in the HPPD protein between the KSR and NER can be explained because plants in the KSR population are more uniform with their response to mesotrione, while NER is a field collected population segregating and exhibiting variation in plant to plant response to mesotrione application. Since a polyclonal HPPD antibody was used, non-specific and cross hybridization occurred due to the cross-reactivity of the antibody with other proteins in the sample. In all, our data indicate that the increased mRNA levels observed in the resistant populations are translated into increased protein levels.
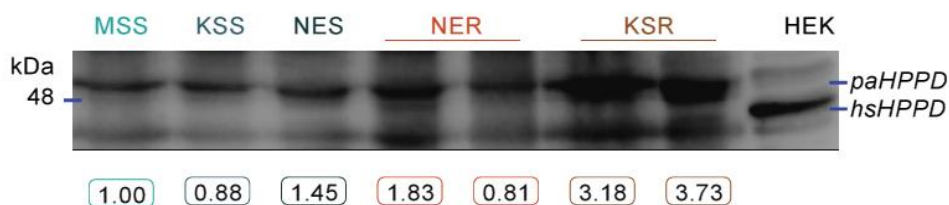
Figure 2.5 Lysates of indicated populations were resolved by SDS-PAGE and immunoblotted for the presence of HPPD in mesotrione-susceptible (MSS, KSS and NES) and -resistant (KSR and NER) Palmer amaranth populations using a rabbit polyclonal antibody against human HPD antibody. For NER and KSR Palmer amaranth, 2 individuals from each population were analyzed. Last lane contains lysate derived from HEK 293 cells (human embryonic kidney cells) and was used as a positive control. Equal amounts of proteins (100 μg) were loaded on gel. The blot was also quantified and MSS is normalized to 1 and other populations were calculated relative to MSS (numbers shown below the blot for each lane).

Discussion

HPPD-inhibiting herbicides are relatively new group of herbicides that effectively control a broad spectrum of broadleaf and some grass weeds. Mesotrione is a triketone developed for pre- and post-emergence control of many broadleaf weeds along with some grass

weeds in corn. To date, only two weeds species, belonging to the same botanical family, Amaranthaceae, have evolved resistance to HPPD-inhibitors, namely waterhemp and Palmer amaranth (Hausman et al., 2011; Thompson et al., 2012; Heap 2016). Plant species can evolve resistance to herbicides essentially via two main mechanisms, a) non-target-site based involving decreased absorption, reduced translocation and/or enhanced metabolism of herbicides and b) target-site based as a result of mutations in the target gene or increased levels of the target protein, enabled through gene amplification or transcriptional upregulation. Absorption and translocation of mesotrione was similar for mesotrione-resistant and -susceptible Palmer amaranth populations in this study (Fig. 2.2) and did not appear to contribute to resistance. The uptake of [^{14}C] mesotrione in Palmer amaranth is consistent and corresponds to the mean absorption of radiolabeled mesotrione across different time points as reported in waterhemp population from IL (Ma et al., 2013). Enhanced detoxification, likely by cytochrome P450 monoxygenases as the mechanism of mesotrione resistance, has been reported in waterhemp population from IL (Ma et al., 2013).

Plants can detoxify both exogenous and endogenous compounds through large family of enzymes known as cytochrome P450 monooxygenases. However, the degree to which each plant can metabolize and degrade xenobiotic chemicals is a major contributor to their survival and in the evolution of resistance. For example, crops like corn, wheat, rice, and sugarcane have a natural tolerance to several groups of herbicides (e.g. HPPD-, ALS-inhibitors) conferred by cytochrome P450 detoxification mechanism (Mitchell et al., 2001; Kreuz et al., 1996). The data presented here suggest that Palmer amaranth resistance to mesotrione results, in part, from the ability to rapidly metabolize this herbicide (Fig. 2.3). Our data also provides a correlation between the rate of mesotrione degradation and the degree of susceptibility or resistance.

Resistant Palmer amaranth (KSR) was able to detoxify 50 % of mesotrione (T_{50} 5.9 h; Fig. 2.6) in a short time compared to corn (T_{50} 11.9 h) and waterhemp (T_{50} 12 h). Similarly, waterhemp susceptible to mesotrione required about 30 h (T_{50}), which is about two times slower than susceptible Palmer amaranth (Ma et al., 2013). However, our data also suggest that the susceptible individuals also completely metabolize mesotrione by 48-72 HAT indicating that detoxification of mesotrione alone may not be the only mechanism of resistance in Palmer amaranth. In weeds, oxidation, hydroxylation or dealkylation of different herbicides, by cytochrome P450s has been reported to be one of the major non-target-site mechanisms confirming resistance to herbicides in both broadleaf and grass weed species (Powles and Yu, 2010).

Recently a rice cytochrome P450 gene, CYP72A31 has been identified to confer resistance to ALS-inhibiting herbicides in both rice and *Arabidopsis* (Saika et al., 2014). Previously Pan et al (2006) reported involvement of rice CYP81A6 in imparting resistance to PS II- and ALS-inhibiting herbicides. Furthermore, when wheat CYP71C6v1 cDNA was cloned and expressed in yeast, AL- inhibiting herbicides were metabolized via phenyl ring hydroxylase (Xiang et al., 2006 a and bb). Transcriptomic analysis of diclofop-resistant rigid ryegrass (*Lolium rigidum*) revealed involvement of three cytchrome P450 genes, a nitronate monooxygenase (NMO), three glutathione S-transferases (GST), and a glucosyl transferase (GT) in detoxification of diclofop (Gaines et al., 2014). However, the specific role of cytochrome P450s in detoxification of mesotrione is unknown and might not suffice to induce resistance. Especially, since it seems to only be temporal difference as all populations are able to fully metabolize mesotrione in 48 hours. Thus, faster degradation of mesotrione alone may not be contributing to the resistance in Palmer amaranth.

In addition to the non-target mechanism of rapid detoxification of mesotrione, the target-site based resistance mechanism(s) such as mutation or amplification of HPPD were also tested in our KSR population. Sequencing of *HPPD* gene did not show any mutations (unpublished) or amplification in this population. On the other hand, we found a significant increase in *HPPD* gene expression (*HPPD* transcript abundance) in mesotrione-resistant populations, suggesting that the resistant plants have a sufficiently high amount of HPPD enzyme available for normal functioning of carotenoid biosynthetic pathway even when exposed to field rate of mesotrione. In the model plant, thale cress (*Arabidopsis thaliana*), constitutive over expression of *HPPD* that was 10-fold higher than the wild type plants showed increased tolerance to sulcotrione, a triketone herbicide (Tsegaye et al., 2002). Similarly, heterologous expression of barley *HPPD* in tobacco also resulted in 10-fold higher resistance to sulcotrione and 2-fold increase in vitamin E content in tobacco seeds (Falk et al., 2003).

Interestingly, a combined resistance through detoxification and target site upregulation has been observed to insecticides in mosquitos. Here, it has been reported that the insects upregulate metabolic enzymes, esterases, glutathione S-transferases or cytochrome P450 monooxygenases through changes/mutations in the cis/trans-acting elements, gene regulation or via amplification of the genes encoding these enzymes (Xianchun Li et al., 2007). For example, in southern house mosquito (*Culex quinquefasciatus*), *CYP9M10* is overexpressed to 260 fold higher in a pyrethroid-resistant strain compared to a susceptible strain via two mechanisms. Two copies of a large fragment of ~100 kb containing the *CYP9M10*, flanked by MITE (a transposable element) of about 0.2 kb upstream of duplicated copies were found. Since only two copies of this cytochrome cannot explain the 260 fold up regulation, the cis-acting and promoter regions were sequenced and it was discovered that there was a cis-acting mutation which

mediated increased expression (Itokawa et al., 2010). Similarly, it is possible that the upregulation of *HPPD* transcript in mesotrione-resistant Palmer amaranth can occur via changes in the cis or trans acting elements or alterations in the promoter region of the *HPPD* gene. To our knowledge, this is the first case of naturally evolved herbicide resistance as a result of increased target-site gene expression without gene amplification. Experiments are in progress in our laboratory to investigate co-segregation of non-target-site based (metabolism) and target-site based (increased *HPPD* gene expression) resistance to mesotrione using forward genetics approach in our Palmer amaranth population.

In addition to herbicide selection pressure, availability of extensive genetic variability, high growth rate and fecundity, adaptation to wide ecological conditions in Palmer amaranth (Knezevic et al., 1997), metabolic resistance and increased *HPPD* gene expression provides an adaptive advantage to survive and spread under diverse environmental stresses. However, the fitness of such herbicide-resistant Palmer amaranth is not known and investigation of fitness costs associated with the resistance trait can help predict the dynamics of evolution and spread of mesotrione resistance in other populations. Furthermore, transcriptome analysis of mesotrione-resistant Palmer amaranth with multiple mechanisms will be a valuable genetic resource: a) to identify and characterize the precise role of specific cytochrome P450s and other target and non-target genes in mesotrione resistance and b) in the research and development of novel herbicides and herbicide tolerant crops.

The mesotrione-resistant Palmer amaranth populations used in this study are also resistant to atrazine (PS II-inhibitor) and chlorsulfuron (ALS-inhibitor), two widely used herbicides in corn production. In general, HPPD-inhibitors are a viable option to manage weeds that are resistant to PS-II and ALS-inhibitors in corn. As Palmer amaranth is a troublesome weed

in corn, evolution of resistance to HPPD-inhibitors in this weed will leave fewer herbicide options for management. As no new herbicide modes of action have been discovered in more than two decades, it is increasingly important to effectively and efficiently use currently available herbicides for sustainable agricultural production. More importantly, the non-target-site based mesotrione resistance in Palmer amaranth may exhibit cross resistance to other known and unknown herbicides that are yet to be discovered. Hence, the weed management strategies in regions with Palmer amaranth and other weeds should include diversified tactics to effectively prevent evolution and spread of multiple herbicide resistance.

Table 2.1 Summary parameters describing the response of MSS and KSS (susceptible) and KSR (resistant) Palmer amaranth aboveground dry biomass to rates of mesotrione 3 WAT. The response was fitted with a three parameter log-logistic model; fitted curves are shown in Figure 2.1.[a]

Population	Regression parameters[b]		GR_{50}[b]	R/S[c]	R/S[d]
	b	*d*	g ai ha^{-1}		
MSS	1.13	100.8 (4.0)	14.9 (1.7)	1	1.76*
KSS	0.95	100.6 (4.8)	8.5 (1.4)	0.6*	1
KSR	0.69	100.9 (4.1)	150.9 (25.9)	10.1**	17.8**

[a] Abbreviations: WAT, wk after treatment; *b*, relative slope around GR_{50}; *d*, upper limit of the response; GR_{50}, mesotrione rate causing 50% reduction in aboveground dry biomass; RS, resistance index (ratio of GR_{50} of MSS or KSS (susceptible) and KSR (resistant) populations.
[b] Values in parenthesis are ± 1 standard error.
[c] RS values based on MSS population.
[d] RS values based on KSS population.
*'**R/S is significantly greater than 1 at P < 0.001 P = 0, repectively.
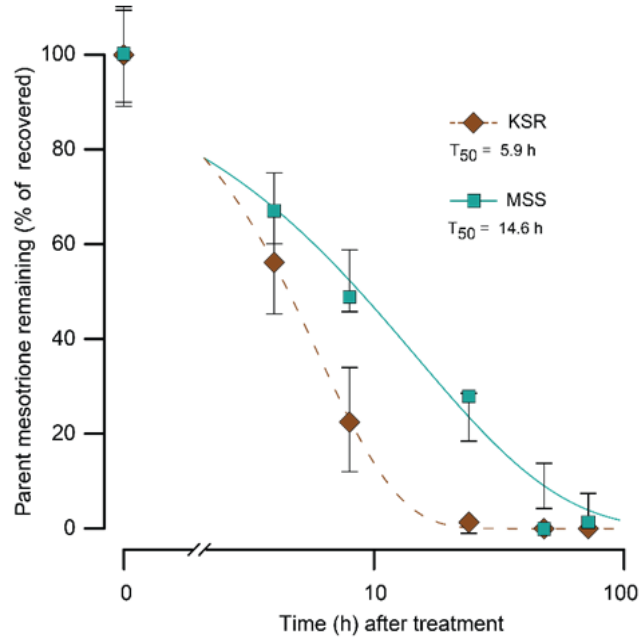
Figure 2.6 (Supplemental) The time course of $[^{14}C]$ mesotrione metabolism (T_{50}) in the treated leaves MSS (susceptible) and KSR (resistant) Palmer amaranth populations across 4, 8, 16, 24, 48 and 72 hours after treatment. Error bars represent the standard error of means of 6-9 biological replicates.

Table 2.2 Summary parameters describing the time course of mesotrione metabolism by MSS (susceptible) and KSR (resistant) Palmer amaranth populations. The response was fitted with a three-parameter Weibull regression; fitted curves are shown in Figure 2.6 (Supplementa1).

Population	Regression parameters[b]		T_{50}[b]	R/S
	b	d	h	
MSS	0.74	99 (5.0)	14.6 (1.9)	1
KSR	1.33	100 (5.0)	5.9 (0.5)	2.45*

[a] b, relative slope around T_{50}; d, upper limit of the response; T_{50}, time taken to metabolise 50% of recovered mesotrione; RS, resistance index (ratio of T_{50} of MSS (susceptible) and KSR (resistant) populations).
[b] Values in parenthesis are ± 1 standard error.
*R/S is significantly greater than 1 at $P < 0.01$.

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Chapter 3 - Atrazine Resistance in Palmer Amaranth (*Amaranthus palmeri*) Endowed by Rapid Detoxification via GST-Conjugation and Not Maternally Inherited

Abstract

Palmer amaranth (*Amaranthus palmeri*) is an economically troublesome, aggressive and damaging weed in the United States and has evolved resistance to six herbicide modes of action including photosystem II (PS II)-inhibitors, such as atrazine. The objective of this study was to investigate the mechanism and inheritance of atrazine resistance in a Palmer amaranth population from Kansas (KSR). The KSR Palmer amaranth showed a high level (178-237 fold more) of resistance to atrazine compared to two known susceptible populations MSS and KSS, from Mississippi and KS, respectively. Nucleotide sequence analysis of the chloroplastic *psbA* gene encoding for D1 protein (atrazine site of action) did not reveal any known mutations conferring resistance to PS II-inhibitors, including the most common serine264glycine substitution specific for triazine resistance. However, the KSR Palmer amaranth rapidly conjugated atrazine via glutathione S-transferase (GST). The time taken for 50 % of atrazine degradation (DT_{50}) was only 0.7 h in KSR compared to 44 h in MSS Palmer amaranth, indicating that KSR population can metabolize atrazine 62 times faster than MSS. Furthermore, genetic analyses of progeny derived from reciprocal crosses of KSR and KSS demonstrated that atrazine resistance in Palmer amaranth is a nuclear trait, supporting no role of maternally inherited *psbA* gene in conferring resistance to atrazine in KSR Palmer amaranth. Here for the first time we show the non-target-site based metabolic resistance to atrazine mediated by GST activity and nuclear inheritance of resistance in KSR Palmer amaranth. This mechanism of atrazine resistance may predispose the Palmer amaranth populations to evolve resistance to multiple herbicides and may spread rapidly, posing a serious threat to management of this weed.

Introduction

Atrazine was introduced in 1958 as a pre- and/or post-emergence herbicide for weed

management in corn and sorghum production. Atrazine belongs to the triazine group of

photosystem II (PS II)-inhibitors, and is used to selectively control annual dicot weeds. PS II-

inhibitors competitively bind to plastoquinone binding (QB) site on the D1 protein in PS II

(Gronwald 1994). Plastoquinone is an important molecule in the electron transport chain that

accepts electrons from PS II and passes on to photosystem I in the process of generating NADPH

and ATP. The maternally inherited chloroplastic gene, *psbA* encodes the D1 protein. Point

mutations in the *psbA* gene will affect binding of atrazine at QB site and can result in the

evolution of resistance to atrazine in weeds (Hirschberg and McIntosh, 1984; Goloubinoff et al.,

1983). A diverse range of mutations in QB site on D1 protein are known in photosynthetic

bacteria and algae which confer resistance to triazines and other chemical classes of PS II-

inhibitors (Oettmeier 1999). The most common target-site mutation conferring triazine resistance

in weeds is serine264glycine substitution in the *psbA* gene, which was first reported in common

groundsel (*Senecio vulgaris* L.) (Ryan 1970). Since then target-site resistance to atrazine has

been documented in many weeds such as kochia (*Kochia scoparia* L.), redroot and smooth

pigweed (*A retroflexus, A. hybidus*), and black nightshade (*Solanum nigrum L.*). Resistance to

PS-II inhibitors is highly prevalent and has been reported in 73 weed species (50 dicots and 23

monocots) across the world (Heap 2016).

The serine264glycine substitution provides high level of triazine resistance and

simultaneously compromises the binding affinity of plastoquinone resulting in reduced

photosynthesis through less efficient electron transport (Gronwald 1994; Arntz et al., 2000). The

target-site based resistance to atrazine is maternally inherited, because the *psbA* is encoded by

the chloroplast genome. The maternal inheritance of triazine resistance is also associated with fitness penalty that varies from weed to weed and also under field or controlled conditions. Triazine-resistant common groundsel and redroot pigweed accumulated relatively low dry biomass compared to susceptible biotypes both under competitive and non-competitive conditions (Conard and Radosevich 1979).

Though the target-site based resistance to triazines is prevalent, non-target-site based resistance has also been reported in weeds, such as velevetleaf (*Abutilon theophrasti*), waterhemp (*Amaranthus tuberculatus*), and black grass (*Alopecurus myosuroides*), rigid rye grass (*Lolium rigidum* Gaud.) as a result of enhanced metabolism of atrazine or simazine via glutathione S transferases (GSTs) or cytochrome P450s activity (Burnet et al., 1993; Gray et al., 1996; Cummins et al., 1999; Ma et al., 2013). Cytochrome P450s and GSTs are multifunctional enzymes that are involved in normal metabolism of the endogeneous substrates in phase I and phase II metabolic detoxification of xenobiotics. In addition, GSTs also play an important role in response to auxin, oxidative stress, lipid peroxidation, and in defense against pathogens (Marrs, 1996). Herbicide selectivity and natural tolerance to triazines in crops such as corn and sorghum is due to rapid metabolism of these herbicides by conjugating with glutathione (GSH), catalyzed by GSTs (Lamoureux et al., 1973; Shimabukuro et al., 1971). GSTs are also known to metabolize and detoxify other herbicide classes such as chloroacetamides and thiocarbamates in many crops and weeds (Gray et al., 1996; Hatton et al., 1996; Breaux 1987).

Palmer amaranth is one of the ten dioecious species in the family amaranthaceae and most aggressive, troublesome and economically damaging weeds in several cropping systems across the United States (Ward et al., 2013). Continued selection pressure resulted in the evolution of resistance to herbicides with six different modes of action, including PS II-inhibitors

(Heap, 2016). Atrazine-resistant Palmer amaranth was first documented in Texas, USA in 1993 and later reported in Kansas in 1995. However, the mechanism of atrazine resistance in Palmer amaranth is unknown. The research is based on the hypothesis that atrazine resistance in Palmer amaranth is the result of rapid conjugation of the herbicide similar to some crops. The objective of this study was to investigate if atrazine resistance in Palmer amaranth evolved as a result of mutation in *psbA* gene, or due to non-target-site based detoxification of atrazine. Additionally, the nuclear or maternal inheritance of atrazine resistance in this weed was also demonstrated in this study.

Materials and Methods

Plant Material and Growth Conditions

Atrazine-resistant Palmer amaranth population from Kansas (KSR) and two known susceptible populations from KS (KSS) and Mississippi (MSS) were used in this study. KSR population was derived by crossing male and female plants of Palmer amaranth which survived atrazine (field rate of 2.2 kg ai ha^{-1}) under greenhouse conditions. The original seed was collected from a field at Stafford County in KS (Thompson et al., 2012) where there was long history of applications of PS II- and ALS-inhibiting herbicides. Seeds of KSR, KSS and MSS Palmer amaranth were germinated in small trays (25 x 15 x 2.5 cm) with commercial potting mixture (Miracle Gro) and transplanted into small pots (6 x 6 x 6.5 cm) when seedlings were 2-3 cm tall under green house conditions (25/20ºC day/night temperature and 15/9 h photoperiod, supplemented with 250 µmol m^{-2} s^{-1} illumination provided with sodium vapor lamps). Palmer amaranth plants (5-6 cm tall) were transferred to a growth chamber maintained at 32.5/22.5ºC, 15/9 h photoperiod, 60-70% relative humidity. Light in the growth chamber was provided by

fluorescent bulbs delivering 550 µmol m^{-2} s^{-1} photon flux at plant canopy level. Plants were watered as needed regularly both under greenhouse as well as growth chamber conditions.

Atrazine Dose Response Assay

The KSR, KSS and MSS Palmer amaranth were grown under greenhouse and growth chamber conditions as described above. Field recommended rate of atrazine (Aatrex®4L, Syngenta, 2.2 kg ai ha^{-1}) was used to determine the frequency of resistant individuals in the KSR population before conducting a dose response assay. The frequency of atrazine resistance in KSR Palmer amaranth population was ~75-80%, indicating that the KSR population was segregation for atrazine resistance (data not shown). For the dose response analysis, when the Palmer amaranth plants were 10-12 cm tall (8-10 leaves), atrazine was applied at 0, 70, 140, 280, 560, 1120, 2240 (1X), 4480, 8960, 13440, 17920 and 22400 g ai h^{-1}, where 1X represents the field recommended rate of atrazine. Required adjuvant, crop oil concentrate (COC, Agridex) at 1% v/v was included in all the treatments. Treatments were applied with a bench-type track sprayer (Generation III, De Vries Manufacturing, RR 1 Box 184, Hollandale, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL) delivering 187 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. Plants were returned to the growth chambers maintained at same conditions within 30 min after treatment. Treatments were arranged in a completely randomized design with six replications and the experiment was repeated three times. Because of segregation for KSR Palmer amaranth population to atrazine resistance or susceptibility, six replications were included for each dose (a total of 18 replications for each dose combined from 3 experiments). Aboveground biomass was harvested 3 weeks after treatment (WAT) and plants packed in paper bags were oven dried at 60°C for a week before measuring dry biomass.

DNA Extraction and *PsbA* Gene Sequencing

Genomic DNA (gDNA) was extracted from fresh leaf tissue (100 mg) collected from KSR Palmer amaranth that survived different doses of atrazine in the above dose response study and also from the susceptible MSS and KSS plants using DNeasy Plant Mini Kit (Qiagen). The quality and quantity of DNA was analyzed using 0.8% agraose gel electrophoresis and nanodrop (Nanodrop 1000 Thermo Scientific), respectively. To amplify the *psbA* gene, PCR reactions were performed using T100^{TM} Thermal Cycler (Bio-Rad Inc., Hercules, CA). Each PCR reaction contained 80 ηg of gDNA, 0.5 µM of forward primer (PsbAF: 5'-CTCCTGTTGCAGCTGCTACT-3') and reverse primer (PsbAR: 5'- TAG AGGGAAGTTGTGAGC-3') (Mengistu et al., 2005) and PCR master mix (Promega). PCR product of known 578 bp in length covering most of the known mutations in the *psbA* gene was amplified using the following PCR conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s and a final extension at 72°C for 7 min. The PCR products were sequenced by GENEWIZ (GENEWIZ Inc.NJ) and the alignment of DNA sequences from KSR and MSS and KSS was performed using MultAlin software (Corpet, 1988).

Atrazine Metabolism in Treated Leaves

The KSR and MSS Palmer amaranth seedlings were grown as described above and moved to the growth chamber to acclimate for 2-3 days before applying [U-^{14}C] atrazine. A total of 20 µl of [U-^{14}C] atrazine consisting of 6.7 k Bq was used to apply on the fourth and fifth youngest leaves (10-12 cm tall plants) as ten 1 µl droplets on each leaf. At 1, 2, 4, 8, 24, 48 and 72 hours after treatment (HAT), treated leaves were harvested and rinsed with 5 ml of wash solution (10% v/v ethanol and 0.5% v/v Tween 20, Thermo Fisher Scientific Inc.) in a 20 mL

scintillation vial for 60 seconds to remove the unabsorbed [U-^{14}C] atrazine. Washed treated

leaves were ground in liquid nitrogen and metabolites of [U-^{14}C] atrazine including the parent

[U-^{14}C] atrazine were extracted by incubating in 15 ml of 90% acetone (HPLC grade, Thermo

Fisher Scientific) at 4°C overnight (16 h). Following overnight incubation, the samples were

centrifuged at room temperature at 6500 rpm, 10 min. Supernatant was collected and evaporated

using a rotary evaporator (Centrivap, Labconoco) for 2-3 h at 45 °C. The supernatant was

concentrated until a volume of 500-1000 μL and centrifuged at high speed (13000 rpm, 10 min,

room temperature). The total extractable radioactivity in each sample was measured by liquid

scintillation spectrometry (LSS) and normalized to 10000 dpm/50μL (0.165 k Bq/50μL) using

50% acetonitrile (HPLC grade, Thermo Fisher Scientific).

Reverse-phase high performance liquid chromatography (HPLC, Beckman Coulter,

System Gold) was used to resolve the total extractable radioactivity into parent [U-^{14}C] atrazine

and its conjugated metabolites using the method similar to and optimized for mesotrione in our

laboratory (Godar et al., 2015). Breifly, reverse-phase HPLC was performed with a Zorbax SB-

C18 column (4.6 x 250 mm, 5-μm particle size; Agilent Technologies) at a flow rate of 1 mL

min^{-1}. Eluent A was water with 0.1 trifluoroacetic acid (TFA, HPLC grade, Thermo Fisher

Scientific) and eluent B, acetonitrile with 0.1% TFA (HPLC grade, Thermo Fisher Scientific).

Radiolabeled compounds were detected with a radioflow detector (EG & G Berthold, LB 509)

and Ultima-Flo M cocktail (Perkin-Elmer). Parent [U-^{14}C] atrazine remaining in each sample

was determined as a percentage of total extractable radioactivity recorded by the peak areas.

Preparation of Synthetic Glutathione (GSH)-Atrazine Conjugate as Standard

As a standard for HPLC analysis of [U-^{14}C] atrazine and its conjugates/polar metabolites,

a synthetic glutathione conjugate of atrazine was produced as described previously for generating

an atrazine-GSH/dimethenamid-GSH conjugate (Ma et al., 2013; Riechers et al., 1996). Briefly, 20 mM of GSH was incubated with 0.1 mM of [U-^{14}C] atrazine in 60 mM 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid (TAPS) buffer (pH 9.5) at 35 °C for 24 h. Only 0.1 mM of [U-^{14}C] atrazine without GSH was incubated in 60 mM TAPS *in vitro* at the same conditions as a positive control reaction. These two reactions were then analyzed by HPLC under above mentioned conditions to determine the retentions times of the GSH-[U-^{14}C] atrazine conjugate and the parent compound of atrazine [U-^{14}C] to analyze the metabolites of atrazine in [U-^{14}C] atrazine treated resistant and susceptible Palmer amaranth.

Inheritance of Atrazine Resistance in Palmer amaranth

The KSR Palmer amaranth plants were grown under greenhouse conditions as described above. When plants reached ~15-20 cm tall, individual plants were multiplied via nodal cuttings to produce vegetative clones. When the clones were established and 10-12 cm tall, half of them were treated with field recommended rate of atrazine (2.2 kg ai ha^{-1}) to determine resistance or susceptibility and rest of the clones were kept for identifying the sex (male or female) and for making crosses. Upon flowering, male and female clones of atrazine-resistant (AR) and atrazine-susceptible (AS) Palmer amaranth were selected. Reciprocal crosses were performed between AR and AS Palmer amaranth (♀AR x ♂AS and ♀AS x ♂AR) by bagging each cross with the pollination bags in the greenhouse to produce F$_1$ seed. Upon germination of F$_1$ seed, the seedlings were grown as described above. Ten-12 cm tall, F$_1$ plants and 10 plants from each cross were treated with two times the field rate (4.4 kg ai ha^{-1}) of atrazine and evaluated for atrazine resistance and susceptibility 2 WAT. The following hypotheses were made before evaluating the F$_1$ progeny response to atrazine treatment:

Hypothesis	Progeny	♀ Atrazine-susceptible (AS) X ♂ Atrazine-resistant (AR)
I	F_1	100% susceptible - maternally inherited
II	F_1	100% resistant or segregating - nuclear inherited

Statistical Analysis

All the experiments were conducted in a completely randomized design and the data from all experiments were combined for each study before performing statistical analysis as there was no interaction between the experiments and treatments. Each experiment has 6-8 replicates. Dose-response data (expressed as percentage of the untreated control) were analyzed using 'drc' package in R 3.1.2 (Knezevic et al., 2007; R Core Team 2015; Ritz and Streibig 2014; Seefeldt et al., 1995). The three parameter log-logistic model as shown below was used to show the relationship between herbicide rate and biomass,

$Y = d/[1+\exp\{b[\log(x)-\log(GR_{50}))]\}]$ where Y is the response (dry biomass or plant health) expressed as percentage of the untreated control, d is asymptotic value of Y at upper limit, b is the slope of the curve around GR_{50} (the herbicide rate giving response halfway between d and the lower asymptotic limit which was set to 0), and x is the herbicide rate. Resistance index (R/S) was calculated as GR_{50} ratio between the MSS or KSS and the KSR populations. Similarly, for the rate of metabolism (DT_{50}) data was analyzed using the Weibull four parameter model.

Results

Atrazine Dose Response Assay

High level of atrazine resistance was confirmed in KSR as shown in Figure 3.1 A and B. The amount of atrazine required to reduce plant growth to 50% (GR_{50}) 3 weeks after treatment (WAT) was 7830 g ai ha^{-1} for KSR, while the GR_{50} of MSS and KSS were 44 and 33 g ai ha^{-1}, respectively, which is 50-68 times less than the field use rate of atrazine (Fig. 3.1 C). KSR population was >178-237 times more resistant than the susceptible populations at the whole plant

level (Fig. 3.1 C). The individuals of KSR that survived atrazine application showed chlorosis

and necrosis symptoms on the older leaves. However, the new growth did not show symptoms 3

WAT.
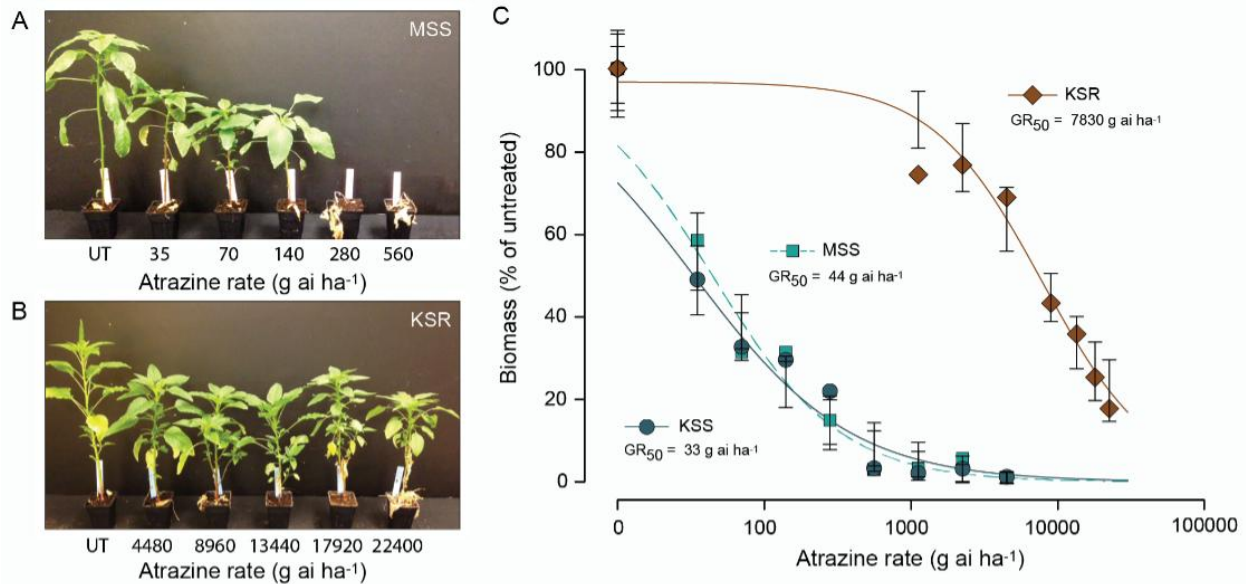
Figure 3.1 Atrazine dose response of susceptible (MSS) and resistant (KSR) Palmer amaranth
populations three weeks after treatment (A and B). (C) Non-linear regression analysis of
aboveground dry biomass of MSS, KSS and KSR populations at different doses of atrazine.
Symbols are averages of 10-13 replicates fitted with a three parameter log-logistic model; model
parameters are shown in Table 3.1.

PsbA Gene Amplification and Sequencing

High level of atrazine resistance in weeds without injury symptoms is most commonly

observed as a result of serine264glycine substitution in the *psbA* gene, encoding the D1 protein

(Gronwald 1994; Preston and Mallory-Smith 2001). To investigate whether alterations in the

psbA gene contribute to the atrazine resistance in Palmer amaranth, we amplified the conserved

fragment (578 bp) of *psbA* gene covering all the known mutations conferring resistance to PS II-

inhibitors from KSR, MSS and KSS plants. A total of 30 KSR (atrazine survived) and 12 MSS

and 6 KSS (untreated) plants were sequenced. Nucleotide sequence comparison of KSR and MSS or KSS did not reveal the most common serine264glycine mutation found in many triazine resistant weed species. The data suggest no amino acid substitutions (Fig. 2), *viz.*, valine219isoleucine, alanine251valine, phenylalanine255isoleucine or asparagine266threonine that are known to confer resistance to other PS II-inhibitors like triazinones and ureas. Single nucleotide polymorphisms encoding identical amino acids were also not observed indicating that the *psbA* gene fragment was highly conserved among the resistant and susceptible Palmer amaranth individuals (Fig. 3.2). Since, we did not find any alterations in the *psbA* gene sequence, we hypothesized that the atrazine resistance may be conferred by a metabolism based resistance mechanism in KSR Palmer amaranth.

Figure 3.2 Nucleotide sequence alignment of *psbA* gene fragment from two atrazine-susceptible (MSS and KSS) and a -resistant (KSR) Palmer amaranth. Nucleotide/amino acid numbering refers to the *Arabidopsis thaliana psbA* gene sequence. Nucleotide polymorphism does not exist between resistant and susceptible Palmer amaranth. Only a few sequences from each population are shown here (3 sequences for each MSS and KSS and 12 for KSR).

Metabolism of [U-^{14}C] Atrazine

Atrazine selectivity, tolerance and resistance in many crops and weed species is conferred by detoxification mediated by GSTs or cytochrome P450 monooxygenases (Lamoureux et al.,

1973; Shimabukuro et al., 1971; Gray et al., 1996). To investigate the possibility of metabolism-based resistance in KSR, [U-^{14}C] atrazine from the treated leaves was resolved into parent [U-^{14}C] atrazine and its polar metabolites over a period of time (1, 2, 4, 8, 24, 48 and 72 HAT). The peak retention times of the parent [U-^{14}C] atrazine and synthetic GSH-[U-^{14}C] atrazine conjugate were observed at 18.7 and 9.7 min respectively (Fig. 3.3). As early as 1, 2 and 4 HAT, between the KSR and MSS significant differences in the amount of parent [U-^{14}C] atrazine and its metabolites were found (Appendix B, Fig. 3.1, 3.2, and 3.3). More than 80-97 % of parent [U-^{14}C] atrazine remained in MSS samples whereas, more than 50-92 % of [U-^{14}C] atrazine was conjugated possibly by GSH in the leaf tissues of KSR Palmer amaranth with in 4 HAT (Fig. 3.3 A and B) because the peak retention time was similar to the synthetic standard of GSH-[U-^{14}C] atrazine conjugate. The amount of parent [U-^{14}C] atrazine remaining in KSR and MSS was 7.5% and 80%, respectively, 4 HAT (Fig. 3.3 C). Interestingly, the amount of parent [U-^{14}C] atrazine decreased to 23% by 48 HAT (Appendix *B*, Fig. 3.6) in the MSS indicating that susceptible plants are very slow in conjugating atrazine and by that time the damage caused to PS II could be irreversible resulting in the injury and death of the susceptible plants.
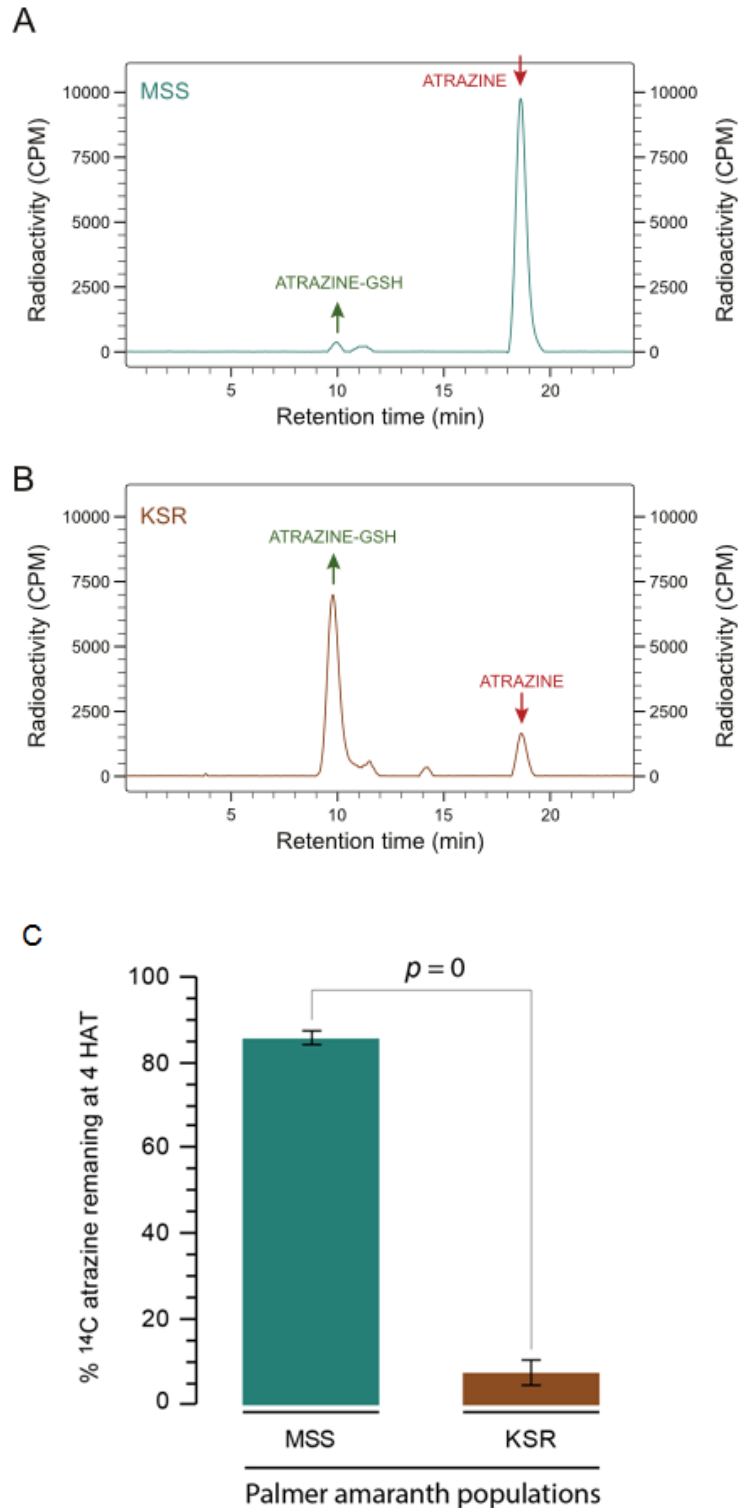
Figure 3.3 Metabolism of [U-¹⁴C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 4 hours after treatment. Reverse-phase HPLC chromatograms of plants treated with [U-¹⁴C] atrazine and analyzed (A) MSS (B) KSS. Peak retention time around 18.7

min is the $[U-^{14}C]$ atrazine (input) and other peak at 9.7 min is the major metabolite of $[^{14}C]$ of atrazine. (C) Represents the amount of $[U-^{14}C]$ atrazine input remaining as percentage of total in the resistant (KSR) and susceptible (MSS) populations 4 HAT. Error bars represent the standard error of means of 5-8 biological replicates.

The rate atrazine metabolism was also determined to estimate the time required to metabolize 50% (DT_{50}) of the parent $[U-^{14}C]$ atrazine. The data showed that there were large and significant differences in the rate of atrazine conjugation by GSTs between the KSR and MSS (Fig. 3.4). The DT_{50} values of atrazine in KSR and MSS were ≤ 0.7 h and greater than 44 h, respectively. This indicates that KSR metabolizes atrazine 63 times faster than MSS. These results provide strong evidence for rapid conjugation of atrazine by GSTs leading to atrazine resistance in KSR Palmer amaranth.
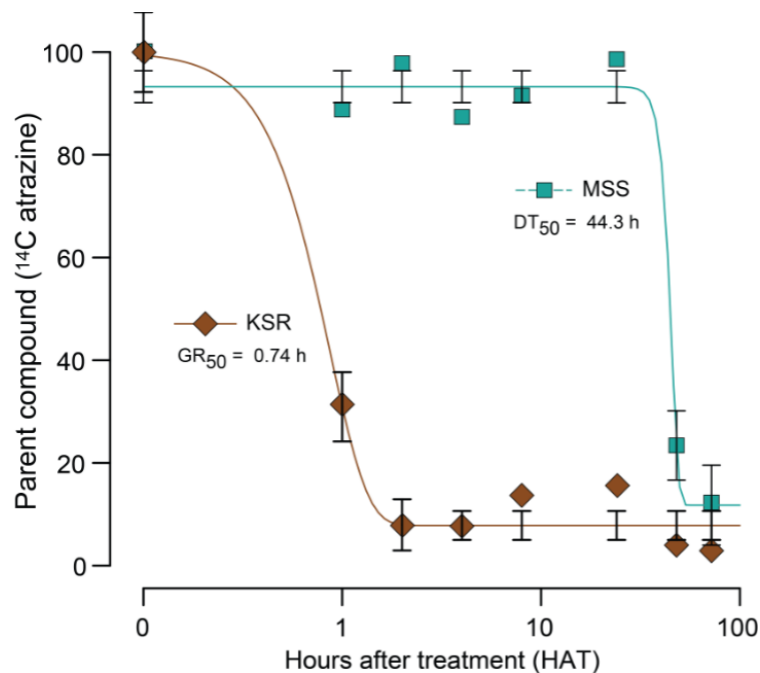
Figure 3.4 The time course of $[U-^{14}C]$ atrazine metabolism (T_{50}) in the treated leaves MSS (susceptible) and KSR (resistant) Palmer amaranth populations across 1, 2, 4, 8, 24, 48 and 72 HAT. Symbols are averages of 5-8 biological replicates fitted with a four parameter Weibull

model; model parameters are shown in Table 3.2. Error bars represent the standard error of means of 5-8 biological replicates.

Inheritance of Atrazine Resistance in Palmer Amaranth

PsbA gene is encoded by the chloroplast genome, which is maternally inherited. Thus, mutations in *psbA* gene resulting in atrazine resistance are expected to inherit only maternally. Since we did not see any mutations in *psbA* gene and additionally, found rapid metabolism of atrazine in KSR Palmer amaranth, we hypothesized that the atrazine resistance may be inherited by a nuclear gene in this population. The response of F_1 progeny generated by reciprocal crosses of AR and AS to atrazine (4.4 kg ai ha^{-1}) showed segregating for atrazine resistance or susceptibility with live and dead plants (data not shown). The hypothesis I is rejected because, if atrazine resistance is a maternally inherited trait, then the progeny generated from cross using AS as a female parent should have all dead upon atrazine treatment. On the other hand, the hypothesis II is accepted, because there was segregation of F_1 progeny from the both reciprocal crosses supporting that the atrazine resistance in KSR Palmer amaranth is controlled by a nuclear gene(s).

Discussion

Herbicide-resistant weeds species across all modes of action have increased dramatically in the last decade and the number of weed biotypes that are resistant to atrazine (73) ranks next only to ALS-inhibitor resistant weeds (159) globally (Heap 2016). However, most cases of weed resistance to atrazine were documented in 1970's and 1980's and decreased gradually in 1990's. Possibly because of the discovery of other effective herbicides, capable of controlling triazine resistant weeds, combined with best management practices such as crop rotation, herbicide rotation, using mixtures of herbicides and other cultural practices. In general, when the resistance

to herbicides is bestowed via target-site based mechanism, with mutations in the herbicide target genes, the plants exhibit high level of resistance with little or no injury. Although, there was no mutation seen in *psbA* gene (Fig. 3.2) of KSR Palmer amaranth, our data from the atrazine dose-response assay showed high level of resistance with little injury (Fig. 3.1A and B). The most common target-site mutation serine264glycine confers resistance to triazines in many weeds such as common groundsel, common lambsquarters (*Chenopodium album*), wild radish (*Raphanus raphanistrum*), kochia and many other weeds (Ryan 1970; Bandeen and McLaren 1976; Friesen and Powles 2007; Varanasi et al., 2015; Heap 2016). A population of common purslane (*Portulaca oleracea*) was found to have serine264threonine substitution and this single amino acid substitution confers resistance to linuron and atrazine, herbicides belonging to two different chemical classes of PS II-inhibitors (Masabni and Zandstra 1999).

The serine264glycine mutation reduces the binding affinity of plastoquinone on D1 protein affecting Hill reaction in photosynthesis and result in reduced rate the carbon dioxide fixation in plants (Holt 1996). With high fitness cost in photosynthesis, the triazine resistant weeds are less competitive compared to the susceptible biotypes and so are present at low frequencies in the weed populations (Jasieniuk et al., 1996). Herbicide-resistant weeds with fitness cost and incidence at low frequencies in the populations have positive implications in controlling resistant weeds. For example, if the atrazine selection is removed and resistance trait is maternally inherited, with fitness penalty, the spread of resistance will be slow and eventually the resistant plants disappear from the population (Jasieniuk et al., 1996). In contrast, the nuclear inheritance of the atrazine resistance in Palmer amaranth facilitate the rapid spread via both seed and pollen. Further crossing and selfing (pseudo) of the F_1 progeny from reciprocal crosses to generate F_2 may provide insights into the number of nuclear genes involved in the resistant trait.

Experiments are in progress in our laboratory to determine the number of nuclear genes controlling atrazine resistance in KSR Palmer amaranth.

The nuclear inheritance of atrazine resistance and absence of any alterations in the *psbA* gene sequence provided evidence that atrazine resistance in Palmer amaranth is mediated by non target-site resistance mechanism. The constitutive expression of glutathione (GSH) and GSTs in corn and sorghum and ability of the plants to induce the expression of these enzymes in response to triazines significantly contribute to the tolerance in crops (Jachetta and Radosevich, 1981; Timmerman 1989). GSTs have also been implicated in herbicide resistance in both broadleaf and grass weeds. Waterhemp and velvetleaf evolved atrazine resistance via GST mediated metabolism based mechanism (Gronwald et al., 1989; Ma et al., 2013). In corn and waterhemp, the DT_{50} of atrazine conjugation were 0.6 and 2.2 h (Ma et al., 2013), respectively, and the KSR Palmer amaranth (Fig. 4) also was able to conjugate atrazine as fast as corn.

In blackgrass (*Alopecurus myosuroides*), both GSTs and P450s are implicated in the multiple herbicide resistance and it was found that specific type I GST named as *Am*GST2 was elevated in resistant blackgrass compared to the susceptible biotype. Interestingly, the activity of *Am*GST2 was limited in detoxifying herbicides but had high activity as a glutathione peroxidase scavenging the hydrogen peroxides formed due to herbicide stress (Cummins et al., 1999). Late watergrass (*Echinochloa phyllopogon*) was resistant to fenoxaprop-*p*-ethyl due to rapid GHS-conjugation of the herbicide (Bakkali et al., 2007). On the other hand, in rigid ryegrass simazine was reported to be detoxified by cytochrome P450s (Burnet et al., 1993). The metabolism results from this study confirm that atrazine resistance in Palmer amaranth is conferred by rapid rate of atrazine conjugation by GSTs (Fig 3.4) similar to atrazine detoxification found in atrazine-resistant waterhemp or corn (Ma et al., 2013). The increased catalytic activity of GST in atrazine

resistant velvetleaf might be due to a mutation in the *GST* gene that improves the herbicide binding and thus the catalytic efficiency of the enzyme (Anderson and Gronwald 1991; Powles and Yu 2010). Another possibility of enhanced detoxification of atrazine in weeds may include increased expression of GST via gene amplification or through gene regulation which is commonly found in insects to insecticide resistance (Enayati et al., 2005) and likely to evolve and select for weeds with new mechanisms of resistance in the future.

Non-target-site based resistance mechanism and nuclear inheritance of atrazine-resistance in Palmer amaranth demonstrated in this study pose a serious threat to sustainable agriculture, especially in no till systems with significant implications. Palmer amaranth may exhibit cross resistance to other known or unknown herbicides, limiting herbicide options for controlling this weed. In addition, atrazine-resistance in Palmer amaranth will spread rapidly via both pollen and seed due to nuclear inheritance. Weed management strategies should include diversified tactics to effectively prevent evolution and spread of multiple herbicide resistance in Palmer amaranth.

Table 3.1 Summary parameters describing the response of MSS and KSS (susceptible) and KSR (resistant) Palmer amaranth aboveground dry biomass to rates of atrazine 3 WAT. The response was fitted with a three parameter log-logistic model; fitted curves are shown in Figure 3.1 C.[a]

Population	Regression parameters[b]		GR_{50}[b]	R/S[c]	R/S[d]
	b	*d*	g ai ha^{-1}		
MSS	0.99	100.8 (4.0)	44 (8.4)	0.6*	1
KSS	0.81	100.6 (4.8)	33 (8)	1	1.76*
KSR	1.1	97 (4.3)	7830 (1146)	237**	178**

[a] Abbreviations: WAT, week after treatment; *b*, relative slope around GR_{50}; *d*, upper limit of the response; GR_{50}, atrazine rate causing 50% reduction in aboveground dry biomass; RS, resistance index (ratio of GR_{50} of MSS or KSS (susceptible) and KSR (resistant) populations.
[b] Values in parenthesis are ± 1 standard error.

*'**R/S is significantly greater than 1 at P < 0.001

Table 3.2 Summary parameters describing the time course of [U-^{14}C] atrazine metabolism by MSS (susceptible) and KSR (resistant) Palmer amaranth populations. The response was fitted with a three-parameter Weibull regression; fitted curves are shown in Figure 3.4.

Population	Regression parameters[b]		T$_{50}$[b]	R/S
	b	*d*	h	
MSS	2.2	93 (1.5)	44 (5.3)	1
KSR	13	100 (3.8)	0.7 (0.4)	62*

[a] *b*, relative slope around T$_{50}$; *d*, upper limit of the response; T$_{50}$, time taken to metabolise 50% of recovered mesotrione; RS, resistance index (ratio of T$_{50}$ of MSS (susceptible) and KSR (resistant) populations).
[b] Values in parenthesis are ± 1 standard error.
*R/S is significantly greater than 1 at P < 0.01.

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Chapter 4 - Target- and Non-Target-Site Based Resistance to ALS-Inhibitors in Palmer Amaranth (*Amaranthus palmeri*)

Abstract

Resistance to acetolactate synthase (ALS)-inhibitor is widespread in many problematic and troublesome weed species, including Palmer amaranth throughout the US due to continuous and repeated selection. However, the precise mechanism of ALS-inhibitor resistance in Palmer amaranth from Kansas (KSR) is not known. The objective of this research was to investigate the physiological and molecular basis of resistance to ALS-inhibitor, chlorsulfuron in KSR. Our results indicate that the KSR population exhibits high level of resistance to chlorsulfuron compared to two known susceptible populations, MSS and KSS from Mississippi and KS, respectively. MSS is highly susceptible to chlorsulfuron whereas KSS is moderately sensitive and dose response analysis revealed that KSR was 275 fold more resistant compared to KSS. Nucleotide sequence analysis of *ALS* gene from the chlorsulfuron survived plants revealed the possibility of evolution of both target- and non-target-site based resistance to ALS-inhibitors in the KSR population. The most common mutation (proline197serine) in *ALS* gene, associated with resistance to sulfonylurea (SU) group of ALS-inhibitors, in many weed species was found only in 30% of KSR population, suggesting that ~70% of plants might have a non-target-site, P450 mediated metabolism based resistance to these herbicides. This is the first report elucidating the mechanism of resistance to ALS-inhibitors in Palmer amaranth from KS. Presence of both target-site and non-target-site based mechanisms of resistance limits the herbicide options to manage Palmer amaranth threatening the sustainable agriculture.

Introduction

Acetolactate synthase (ALS) enzyme catalyzes the indispensable step in the biosynthesis of branched-chain amino acids, valine, leucine, and isoleucine in plants and microorganisms (Shaner 1991; Dailey and Cronan 1986). ALS enzyme is the target-site of several herbicide classes in the group of ALS-inhibitors such as sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinylthiobenzoates (PTB) and sulfonylaminocarbonyltriazolinones (SCT). Differential selectivity of ALS-inhibitors enabled its widespread use in many crops including wheat (*Triticum aestivum* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* (L.) to control broad spectrum of grass and broadleaf weeds (Beyer et al., 1998). ALS-inhibitors are also popular because of their low dose, high efficacy, low mammalian toxicity and low cost. Extensive and widespread use of these herbicides created a strong selection pressure resulting in the evolution of herbicide resistance in a number of weed species. Sulfonylurea herbicide resistance was reported as early as 1987 in prickly lettuce (*Lactuca serriola*) in no-till winter wheat in Idaho (Mallory-Smith et al., 1990) and since then 159 weed species have evolved resistance to ALS-inhibitors including many *Amaranthus* species (Heap 2016).

Palmer amaranth (*Amaranthus palmeri*) has evolved resistance to a number of widely used herbicides in different herbicide modes of action including acetolactate synthase (ALS)-, microtubule-, photosystem II (PS II)-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)- and hydroxyphenylpyruvate dioxygenase (HPPD)- and protoporphyyrinogen (PPO)-inhibitors across United States (Heap 2016). The most common mechanism of resistance to ALS-inhibitors in weed species is the target-site based, conferred by point mutations resulting in single amino acid substitutions in the *ALS* gene (Shaner 1999). Predominantly, high level of resistance is

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conferred by these alterations due to reduced sensitivity of the target enzyme to the herbicides. The number of mutations leading to target-site alterations have increased dramatically in last decade and a total of 26 amino acid substitutions were identified across eight amino acid positions (Yu and Powles 2013; Tranel et al., 2016). The proline197, amino acid position on *ALS* gene, has been found to be most commonly prone residue to point mutations. So far, 11 substitutions at this position have been reported in ALS-inhibitor-resistant weeds such as giant ragweed (*Ambrosia trifida*), Kochia (*Kochia scoparia*) and many pigweeds populations, e.g. waterhemp (*A. tuberculatus*), redroot piweed (*A. retroflexus*), smooth pigweed (*A. hybridus*) (Guttieri et al., 1995; Foes et al., 1999; Patzoldt and Tranel 2002; Varanasi et al., 2015).

Non target-site mechanism of resistance, endowed by metabolism was also identified in many ALS-inhibitor-resistant grasses such as rigid ryegrass (*Lolium rigidum*), and broadleaf weeds like wild mustard (*Sinapsis arvensis*) and waterhemp (Christopher et al., 1991; Cotterman and Saari1992; Veldhuis et al., 2000; Guo et al., 2015). Many ryegrass populations (SLR31 and VLR69) from Australia that are resistant to multiple herbicides showed cross resistance to SU (e.g. chlorsulfuron) and IMI (e.g. imazamethabenz) herbicides (Heap and Knight 1986; Christopher et al., 1991). The non-target site based mechanism of resistance to chlorsulfuron has been reported as a result of rapid metabolism of chlorsulfuron by cytochrome P450 monooxygenases (Christopher et al., 1994). Natural tolerance and selectivity to ALS-inhibitors in crops, such as wheat, barley, rice, corn and soybeans is also bestowed because of rapid detoxification of the ALS-inhibitors via wide array of cytochrome P450 enzymes (Brown 1990).

The ALS-inhibitor resistant Palmer amaranth was first documented in Kansas in 1993 (Horak and Peterson 1995) and later reported in many states across the USA. However, the precise mechanism of resistance is not unknown. The research was based on the hypothesis that

ALS-inhibitor resistance in Palmer amaranth may be due to target-site or non-target-site based mechanis. The objective of this study was to determine the molecular basis of chlorsulfuron, a SU herbicide resistance in Palmer amaranth from KS and investigate the cross resistance to other chemical classes of ALS-inhibitors.

Materials and Methods

Plant Material and Growth Conditions

Chlorsulfuron-resistant Palmer amaranth population collected from a field at Stafford County in KS (Thompson et al., 2012) designated as KSR and two susceptible populations from KS (KSS) and Mississippi (MSS) were used in this research. Seeds of KSR, KSS and MSS Palmer amaranth were germinated in small trays (25 x 15 x 2.5 cm) with commercial potting mixture (Miracle Gro) and transplanted into small pots (6 x 6 x 6.5 cm) when seedlings were 2-3 cm tall under green house conditions (25/20 °C day/night temperature and 15/9 h photoperiod, supplemented with additional 250 µmol m^{-2} s^{-1} illumination provided with sodium vapor lamps). Palmer amaranth plants (5-6 cm tall) were transferred to a growth chamber maintained at 32.5/22.5 °C, 15/9 h photoperiod, 60-70% relative humidity. Light in the growth chamber was provided by fluorescent bulbs delivering 550 µmol m^{-2} s^{-1} photon flux at plant canopy level. Plants were watered as needed regularly both under greenhouse as well as growth chamber conditions.

Dose Response Assay

The populations of KSS, MSS and KSR Palmer amaranth were grown as described above and 10-12 cm tall plants were treated with field recommended rate of chlorsulfuron (Glean[®] XP, Dupont Crop Protection, 18 g ai ha^{-1}) to determine the frequency of resistant individuals in the KSR population before conducting the dose response assay. The frequency of chlorsulfuron

resistance in KSR Palmer amaranth population was ~70-75%, indicating that the KSR population was segregating for chlorsulfuron resistance or susceptibility (data not shown). For the dose response analysis, 10-12 cm tall Palmer amaranth plants at 8-10 leaves stage were treated with the following doses of chlorsulfuron 0, 0.056, 1.12, 2.25, 4.5, 9,18 (1X), 36, 72, 108, 144 g ai h^{-1} where 1X represents the field recommended rate of chlorsulfuron. Non-ionic surfactant (NIS) at 0.25% v/v was used as an adjuvant in all the treatments. Herbicide treatments were applied with a bench-type sprayer (Research Track Sprayer, Generation III, De Vries Manufacturing, RR 1 Box 184, Hollandale, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL) delivering 168 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. After treatment, plants were moved back to the growth chamber conditions as mentioned before. Treatments were arranged in a completely randomized design with six replications and the experiment was repeated three times. Aboveground biomass was harvested 3 weeks after treatment (WAT) and plants packed in paper bags were oven dried at 60°C for a week before measuring dry biomass.

Statistical Analysis

Data from all experiments were combined for each study before performing statistical analysis as there was no interaction between the experiments and treatments was found. Dose-response data (expressed as percentage of the untreated control) were analyzed using 'drc' package in R 3.1.2 (Knezevic et al., 2007; R Core Team 2015; Ritz and Streibig 2014; Seefeldt et al., 1995). The three parameter log-logistic model as shown below was used to show the relationship between herbicide rate and biomass,

$Y = d/[1+\exp\{b[\log(x)-\log(GR_{50}))]\}]$ where Y is the response (dry biomass or plant health), expressed as percentage of the untreated control, d is asymptotic value of Y at upper limit, b is

the slope of the curve around GR_{50} (the herbicide rate giving response halfway between d and the lower asymptotic limit which was set to 0), and x is the herbicide rate. Resistance index (R/S) was calculated as the ratio of GR_{50} of KSS and KSR populations.

Screening for Cross Resistance

The KSR and MSS Palmer amaranth seedlings were grown under greenhouse conditions as described above. To determine cross resistance of KSR, to other classes of herbicides in ALS-inhibitor group, the plants were treated with field recommended rates of thifensulfuron (Harmony[®], Dupont Crop Protection), imazamox (Beyond[®], BASF Ag Products), propoxycarbazone (Olympus[®], Bayer CropScience) and pyrithiobac (Staple[®], Dupont Crop Protection) which belong to SU, IMI, SCT and PTB classes, respectively. The field recommended rates of thifensulfuron, imazamox propoxycarbazone and pyrithiobac are 36, 35, 44 and 73 g ai ha^{-1}, respectively. All the treatments included NIS at 0.25% v/v as adjuvant and sprayed using the bench-type sprayer as described before. Plant survival was assessed 3-4 WAT to determine the resistance of KSR Palmer amaranth to each herbicide.

DNA Isolation and *ALS* Gene Sequencing

Fresh leaf tissue was collected from KSR plants that survived different doses of chlorsulfuron in whole-plant dose response experiments and also susceptible (KSS and MSS) Palmer amaranth. The collected tissue (100 mg) was flash frozen in liquid nitrogen and genomic DNA (gDNA) was extracted using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. DNA was quantified using nanodrop (Nanodrop 1000, Thermoscientifc) and quality was analyzed using 0.8% agraose gel electrophoresis. Forward (ALSF: 5'-TCCTCGCCGCCCTCTTCAAATC-3') and reverse (ALSR: 5'-CAGCTAAACGAGAGAACGGCCAG-3') primers were used to amplify *ALS* gene of ~2000 bp

in length (GenBank Accession U55852; Whaley et al., 2007). PCR reactions were performed

using T100^{TM} Thermal Cycler (Bio-Rad Inc., Hercules, CA). Each PCR reaction contained 80-

100 ηg of gDNA, 0.5 µM of forward and reverse primers each and PCR master mix (Promega).

The PCR product was purified using GENEjet PCR purification kit (Thermofisher Scientific)

following the manufacturer's instructions and sequenced using the ALSF and ALSR primers at

GENEWIZ sequencing facility (GENEWIZ Inc. NJ) and the alignment of DNA sequences from

KSR and MSS and KSS was performed using MultAlin software (Corpet 1988).

Results

Chlorsulfuron Dose Response Assay

The result of dose-response assay confirmed very high level of resistance to chlorsulfuron

in the KSR when compared to MSS or KSS Palmer amaranth (Fig. 1). The shoot dry weight

relative to the untreated control measured 3 WAT gradually decreased with increasing rate of

chlorsulfuron, however the KSR plants survived 8 times the field rate of chlorsulfuron (Fig. 1C).

The MSS or KSS Palmer amaranth responded differently to chlorsulfuron application. The MSS

population showed high level of sensitivity and was not able to survive even the lowest dose

(0.56 g ai ha^{-1}) used, which is 32 times less than the field recommended rate (Fig. 4.1 A). On the

other hand the KSS population survived one-fourth of field recommended dose and was

completely killed at half of the field rate (Fig. 4.1B). The amount of chlorsulfuron required to

reduce plant growth to 50% (GR$_{50}$) 3 weeks after treatment (WAT) was 303 g ai ha^{-1} for KSR,

while the GR$_{50}$ of KSS was 1.08 g ai ha^{-1} (Fig. 2). Overall, the KSR population was >275 times

more resistant to chlorsulfuron relative to the KSS Palmer amaranth at the whole plant level (Fig.

2). Since the MSS population was completely killed at very low dose used, the dose-response

curve could not be fitted with KSS and KSR to analyze the GR$_{50}$ value (Fig. 1A).
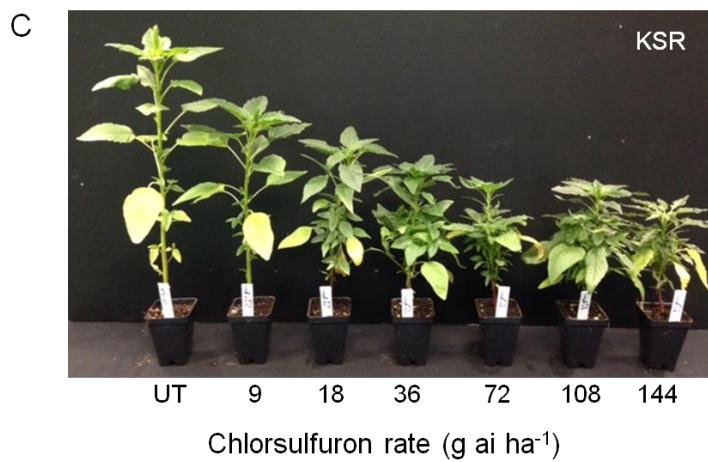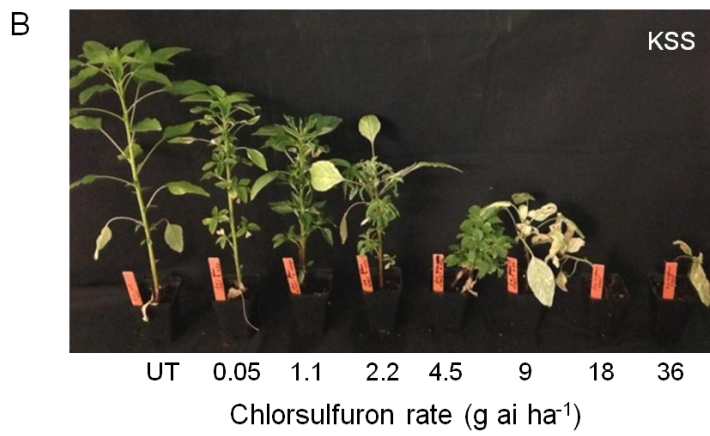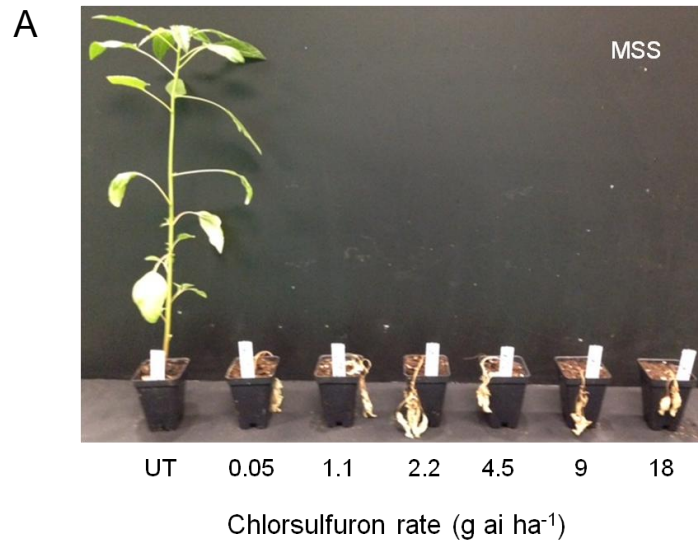
Figure 4.1 Whole plant response of susceptible and resistant Palmer amaranth populations to different doses of chlorsulfuron, 3 weeks after treatment WAT (A) MSS (B) KSS and (C) KSR.
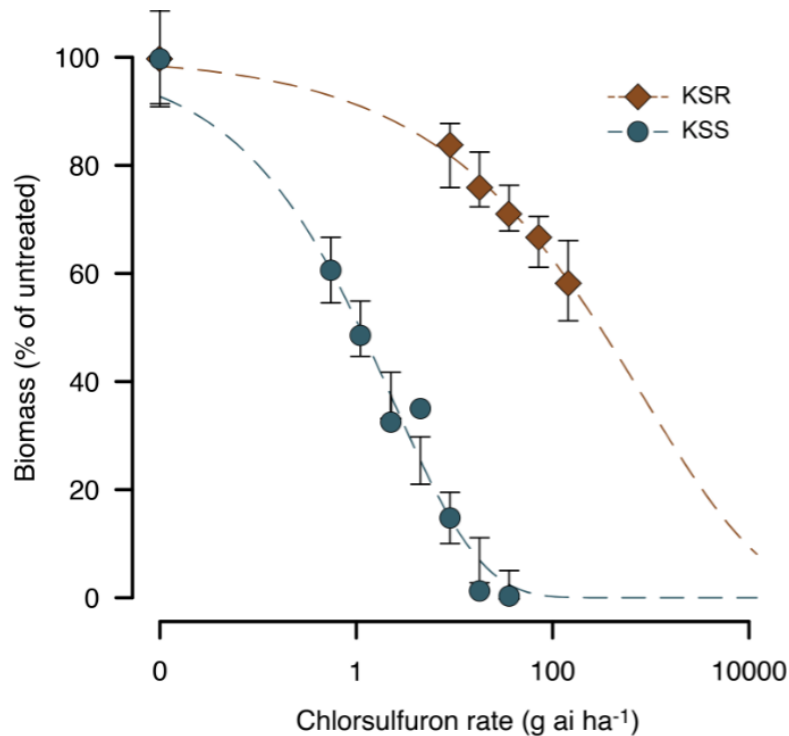
Figure 4.2 Non-linear regression analysis of aboveground dry biomass of KSS and KSR Palmer amaranth to different doses of chlorsulfuron 3 WAT. Symbols are averages of 12-13 replicates fitted with a three parameter log-logistic model; model parameters are shown in Table 4.1.

Molecular Basis of Resistance

High levels of chlorsulfuron resistance observed in the dose response experiments indicated that the resistance may possibly have evolved due to an alteration in the molecular target-site of the ALS-inhibitors (*ALS* gene). To investigate whether mutations in the *ALS* gene contribute to the chlorsulfuron resistance in Palmer amaranth, *ALS* gene (~ 2 kb) was amplified from 30 KSR (chlorsulfuron survived) and 12 MSS and 3 KSS covering all know mutations at eight codon positions. Nucleotide sequence alignment from all the individuals showed interesting results. In approximately 30% of KSR plants, the sequence analysis showed only a single nucleotide polymorphism resulting in an amino acid substitution, proline (CCC) to serine (TCC) at position 197 when compared to MSS or KSS (Fig. 4.3). Other 70% KSR plants did not show any substitutions at all the known codon positions on *ALS* gene, (numbered relative to the ALS

amino acid sequence of *Arabidopsis thaliana*) (Fig. 4.3). These results suggest that existence of

non-target-site, in addition to target-site resistance mechanism in KSR Palmer amaranth to ALS-

inhibitors.

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                                              PRO197SER           |                 ALA 205
ALS MSS-01   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTC CAA GAG ACT
ALS MSS-02   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS MSS-03   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSS-01   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSS-02   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTC CAA GAG ACT
ALS KSS-03   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSR-01   CCG CTT GTC GCC ATT ACT GGG CAA GTT TCC CGG CGT ATG ATT GGT ACT GAT GCT TTC CAA GAG ACT
ALS KSR-02   CCG CTT GTC GCC ATT ACT GGG CAA GTT TCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSR-03   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSR-04   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSR-05   CCG CTT GTC GCC ATT ACT GGG CAA GTT TCC CGG CGT ATG ATT GGT ACT GAT GCT TTC CAA GAG ACT
ALS KSR-06   CCG CTT GTC GCC ATT ACT GGG CAA GTT TCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSR-07   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTC CAA GAG ACT
ALS KSR-08   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSR-09   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
ALS KSR-10   CCG CTT GTC GCC ATT ACT GGG CAA GTT TCC CGG CGT ATG ATT GGT ACT GAT GCT TTC CAA GAG ACT
ALS KSR-11   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTC CAA GAG ACT
ALS KSR-12   CCG CTT GTC GCC ATT ACT GGG CAA GTT CCC CGG CGT ATG ATT GGT ACT GAT GCT TTT CAA GAG ACT
```

Figure 4.3 Nucleotide sequence alignment and analysis of a portion of *ALS* gene sequence from
KSS, MSS and KSR Palmer amaranth populations. Only few sequences from each population
are shown (3 from each MSS and KSS and 12 from KSR). Red text in the nucleotide sequence
codes for proline and green for serine. Nucleotide/amino acid numbering refers to the
Arabidopsis thaliana, *ALS* gene sequence

Cross Resistance to ALS-inhibitors

Lack of known mutations in some of KSR Palmer amaranth provides strong indication

for the existence of non-target-site resistance mechanism of ALS-inhibitor resistance in this

population. Presence of non-target-site based mechanism is known to confer cross resistance to

other chemical classes of ALS-inhibitors similar to some target-site mutations. We investigated

the cross resistance of KSR Palmer amaranth to thifensulfuron, imazamox, propoxycarbazone

and pyrithiobac at field recommended rates. The results suggest that KSR Palmer amaranth was

also resistant to thifensulfuron, propoxycarbazone and pyrithiobac but not imazamox. The

frequency of KSR Palmer amaranth to thifensulfuron, propoxycarabzone or pyrithiobac was ~70-

75% similar to chlorsulfuron (data not shown). These results suggest that non target-site mechanism probably via cytochrome P450-mediated detoxification may contribute resistance to SUs, SCTs and PBTs in KSR Palmer amaranth. Experiments are progress in our laboratory to elucidate the non-target based ALS-inhibitor resistance in Palmer amaranth.

Discussion

The highest number of weed species was documented to have evolved resistance to ALS-inhibitors globally till date (Heap 2016). The most common mechanism of resistance to ALS-inhibitors was identified as a result of mutation in the *ALS* gene, and this type of resistance provides high magnitudes of resistance. Many *Amaranthus* species such as smooth pigweed and waterhemp evolved resistance to ALS-inhibitors due to amino acid substitutions at alanine122threonine, aspartate376glutamate, trptophan574leucine, or serine653threonine and showed varying levels of resistance ranging from 60-3200 fold depending on the type of substitution (Patzoldt and Tranel 2007; Whaley et al., 2006; 2007). Aspartate376glutamate substitution is known to confer a broad spectrum of resistance to all five chemical classes of ALS-inhibitors (SU, IMI, SCT, TP and PTB) (Whaley et al., 2007). Imazethapyr-resistant Palmer amaranth from Kansas (Clay County) was found to be approximately 2800 times more resistant compared to sensitive biotype and was also shown cross resistant to the sulfonylurea herbicides, thifensulfuron and chlorimuron (Sprague et al., 1997). Palmer amaranth from Stafford County in KS (KSR) used in this study also showed high level of resistance to SUs (Fig. 1 and 2) with cross resistance to SCTs and PTBs, however, was susceptible to imazamox. In contrast, IMI resistant Palmer amaranth from Arkansas was cross resistant to chlorimuron, diclosulam and pyrithiobac (Burgos et al., 2001).

ALS gene sequence analysis revealed the presence of both target- and non-target-site resistance mechanisms in KSR Palmer amaranth. Among all the known amino acid substitutions at proline197 resisdue, only serine was found in KSR population. Proline197serine mutation predominantly confers resistance to SUs with no cross resistance to other ALS-inhibitors. Previously, though ALS enzyme inhibition assay in Palmer amaranth indicated that resistance is due to insensitive ALS enzyme (Sprague et al., 1997), our study provides precise evidence for target-site alteration (proline197serine) conferring resistance to sulfonylurea herbicides in this weed. Nonetheless, the majority (~70%) of the KSR plants that are resistant to chlorsulfuron did not show any known mutations at eight codons (Fig. 3.3) indicating that a non-target-site based mechanism. Metabolism based ALS-inhibitor resistance has been reported in rigid ryegrass, blackgrass, rigid brome, wild oat, late watergrass and wild mustard (Yu and Powles 2014). Recently, a waterhemp population from Illinois also showed broad resistance to four chemical classes of ALS-inhibitors through metabolism-based non-target-site resistance mechanism (Guo et al., 2015).

The occurrence of both target site and non target site resistance mechanisms in the same population of a broadleaf weed species is on the rise and usually is masked by target-site resistance. The two other weeds species with a combination of both target and non-target-site based resistance to ALS-inhibitors include corn poppy (*Papaver rhoeas*) (Délye et al., 2011), and waterhemp (Guo et al., 2015). In conclusion, evolution of target and non-target based resistance in weed species poses a serious threat to weed management, as such resistance predisposes weeds to evolve resistance to herbicides with multiple modes of action. More importantly, the dioecious nature of Palmer amaranth contributing to high genetic variability, combined with high seed production, and efficient pollen and seed distribution (Lovell et al., 1996) facilitate the

evolution of resistance to other most commonly used herbicides. Thus management of this

economically important weed is a challenge. Stringent management strategies such as reducing

the selection pressure of herbicides by rotating herbicides as well as crops, and planting weed

free crop, and possibly tillage are warranted.

Table 4.1 Summary parameters describing the response of KSS (susceptible) and KSR (resistant) Palmer amaranth aboveground dry biomass to rates of chlorsulfuron 3 WAT. The response was fitted with a three parameter log-logistic model; fitted curves are shown in Figure 4.2.[a]

Population	Regression parameters[b]		GR_{50}[b]	R/S[c]
	b	d	g ai ha^{-1}	
KSS	0.35	99.7 (4.5)	1.1 (0.2)	1
KSR	0.48	100.2 (4.4)	303 (156)	275*

[a] Abbreviations: WAT, wk after treatment; b, relative slope around GR_{50}; d, upper limit of the response; GR_{50}, mesotrione rate causing 50% reduction in aboveground dry biomass; RS, resistance index (ratio of GR_{50} of MSS or KSS (susceptible) and KSR (resistant) populations.
[b] Values in parenthesis are ± 1 standard error.
[c] RS values based on KSS population.
*'**R/S is significantly greater than 1 at P < 0.001 P = 0, repetively.

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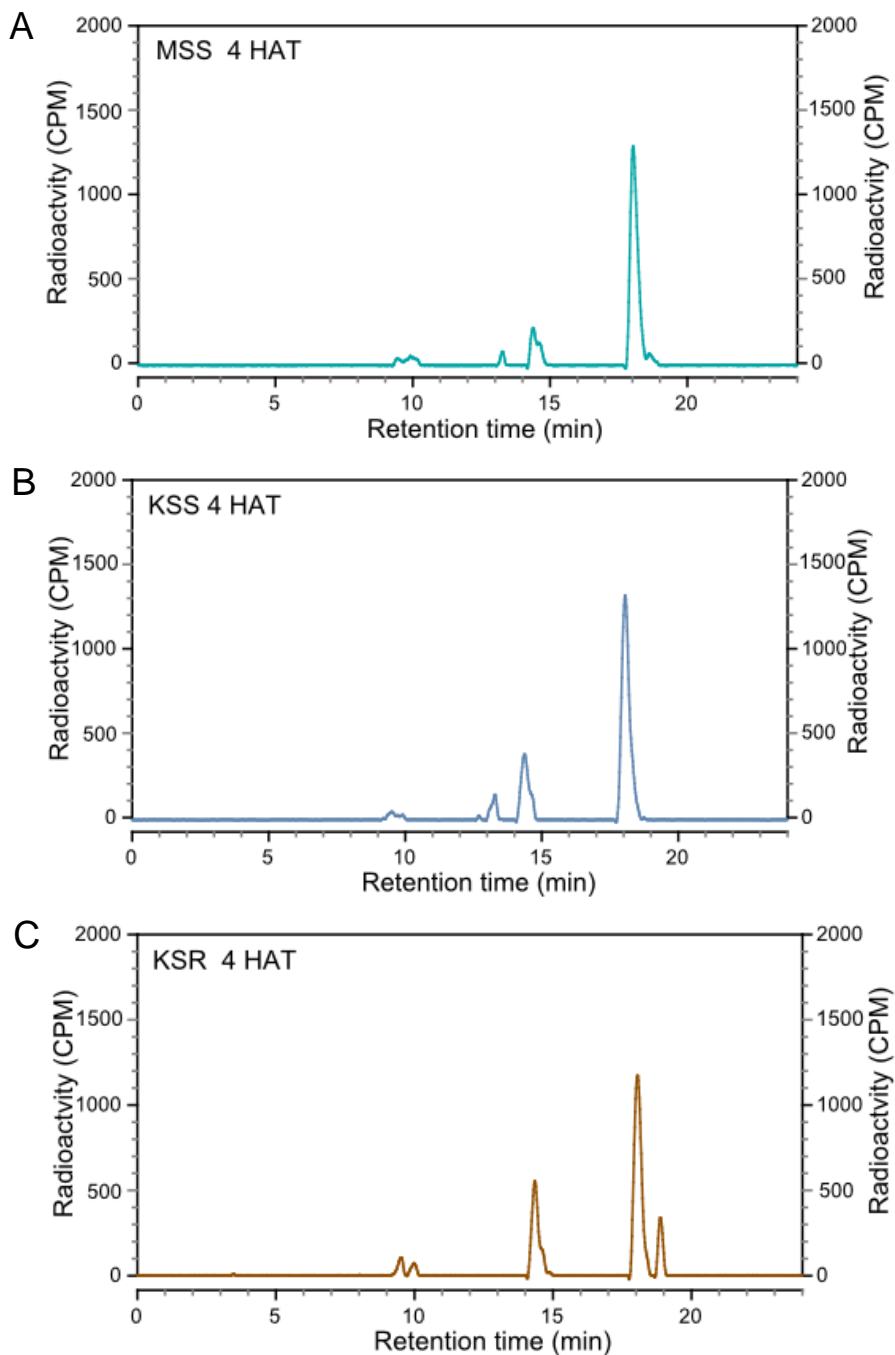
Figure 2.1. Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 4 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested. (A) MSS (B) KSS and (C) KSR. Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione.
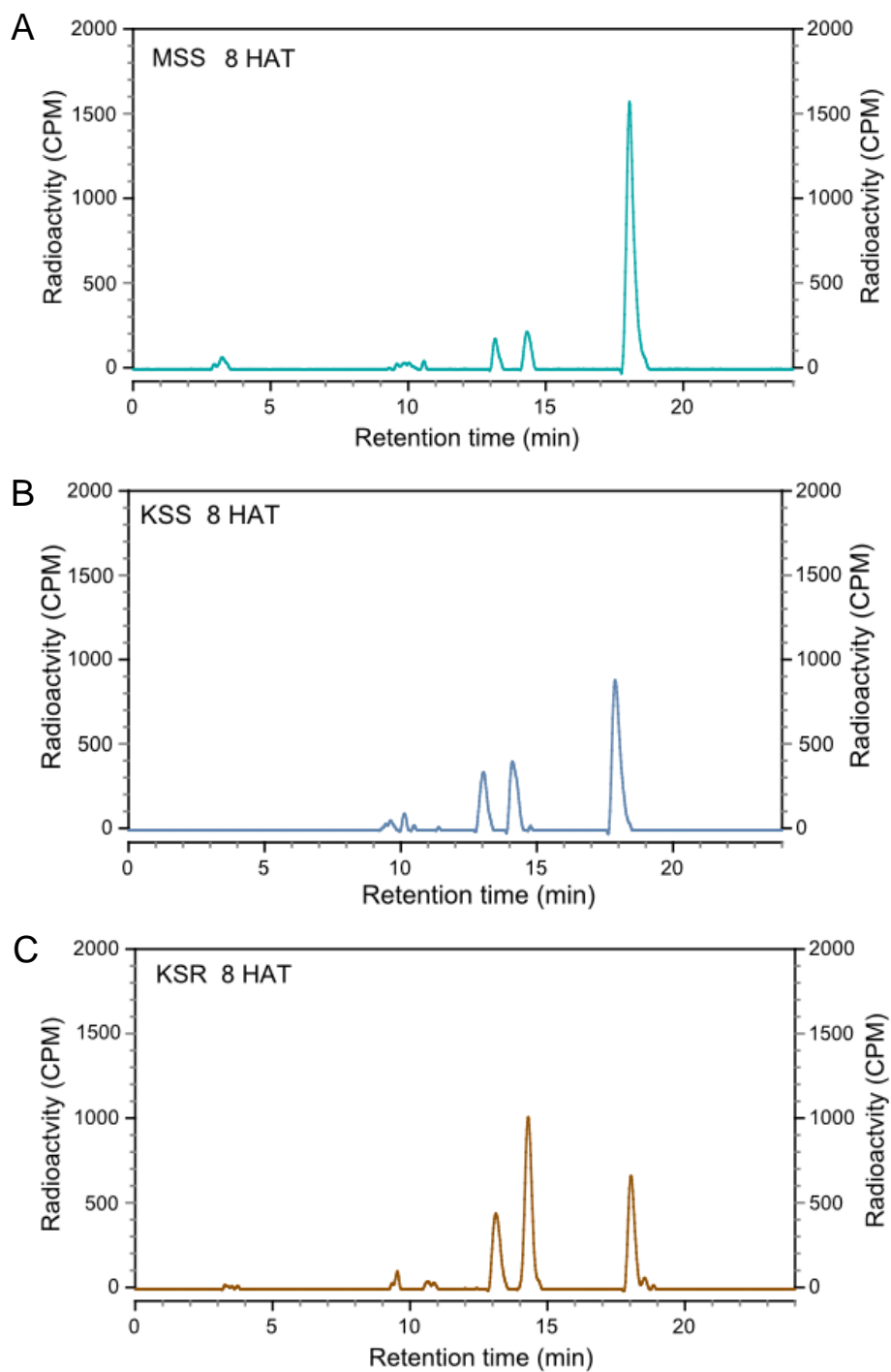
Figure 2.2. Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 8 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested. (A) MSS (B) KSS and (C) KSR. Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione.
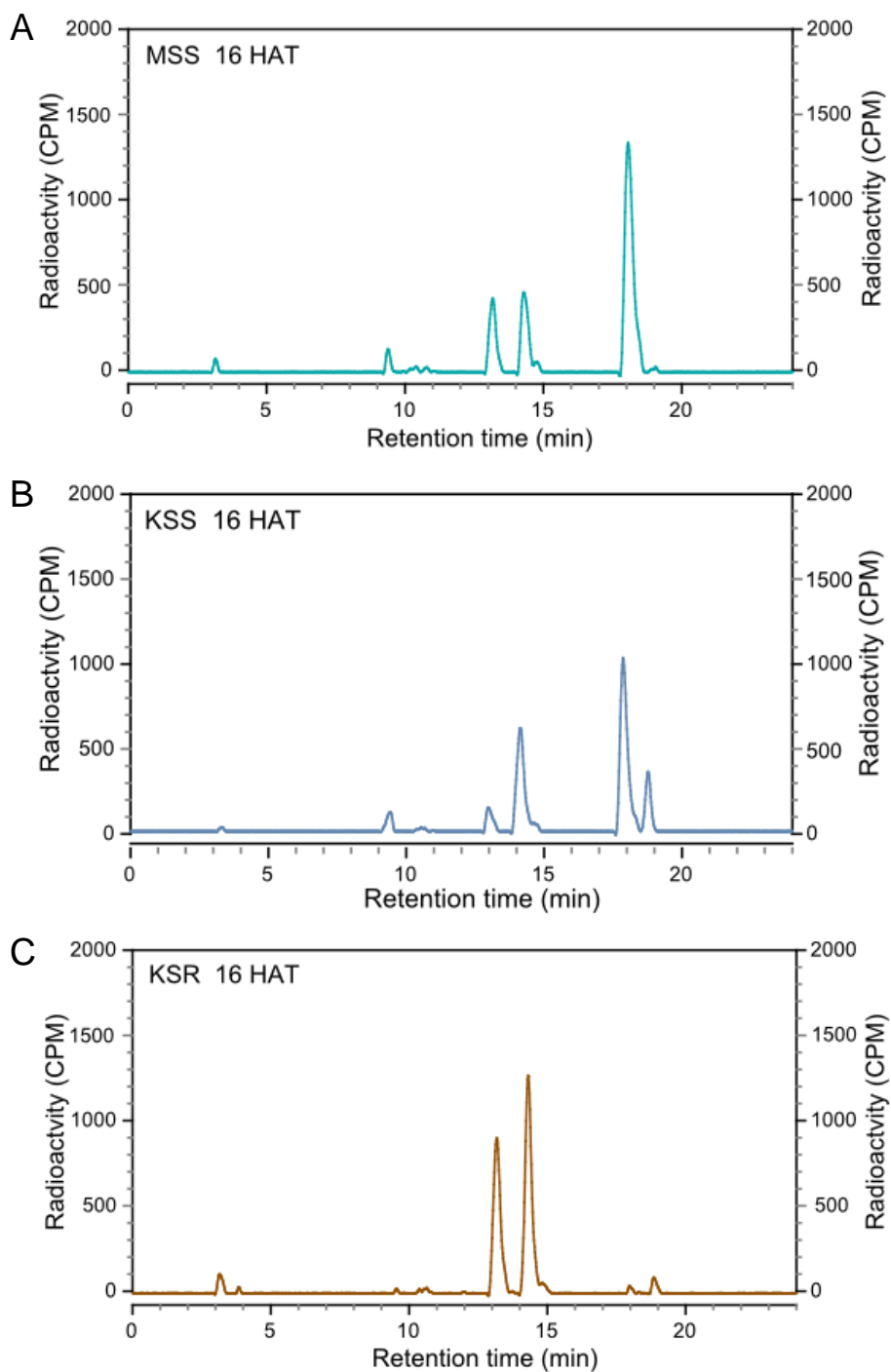
Figure 2.3. Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 16 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested. (A) MSS (B) KSS and (C) KSR. Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione.
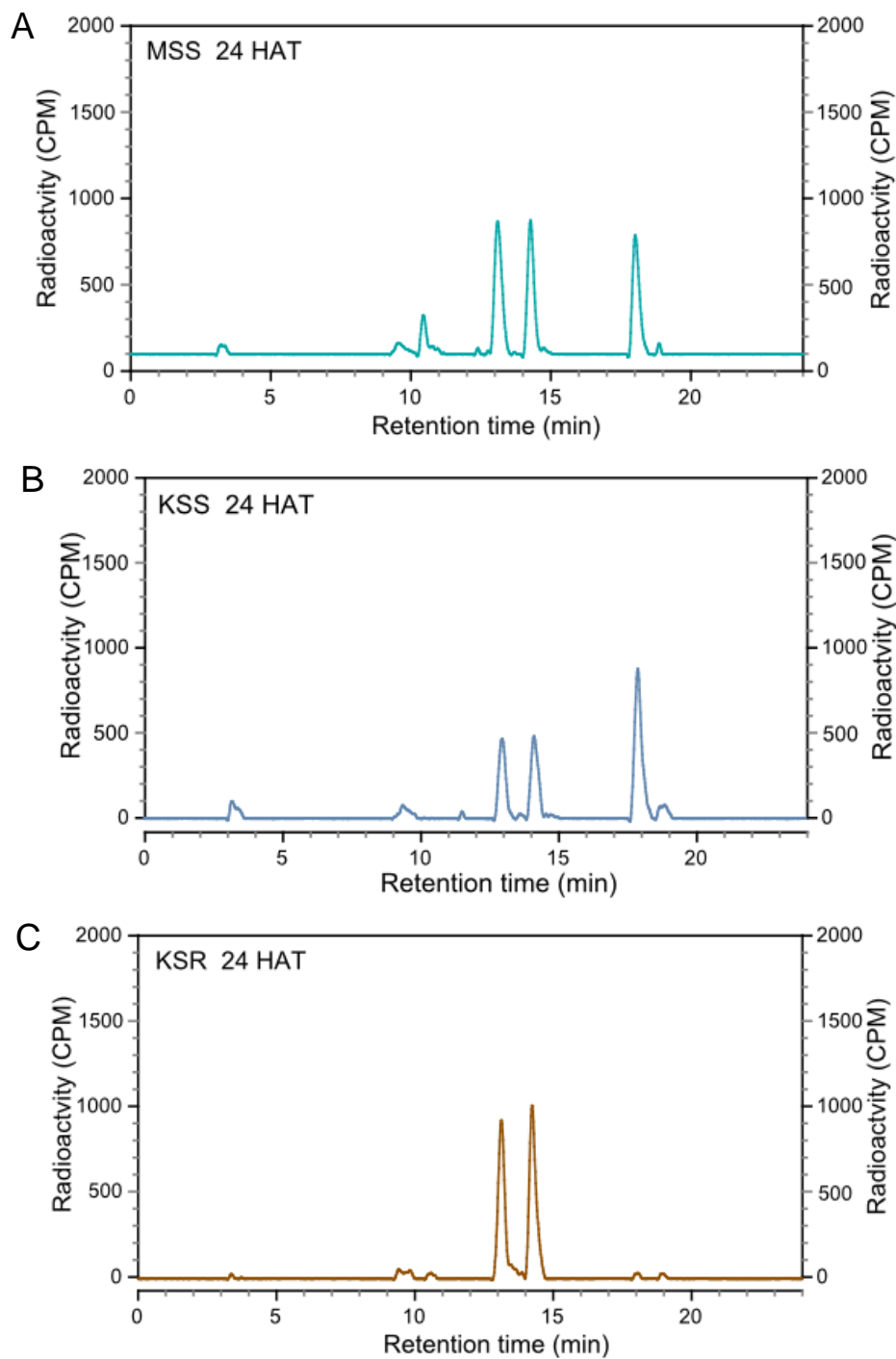
Figure 2. 4. Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 24 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested. (A) MSS (B) KSS and (C) KSR. Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione.
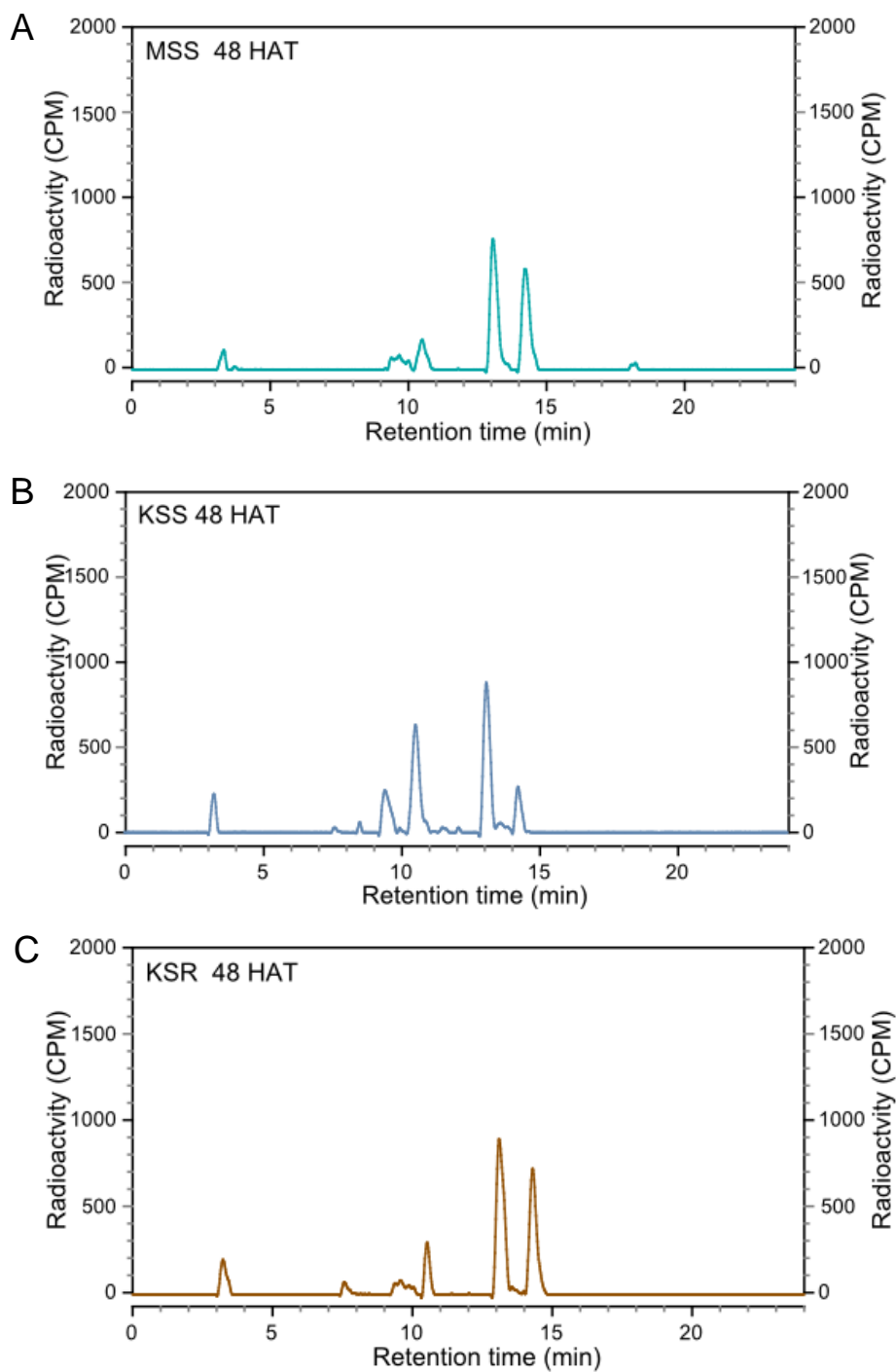
Figure 2.5. Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 48 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested. (A) MSS (B) KSS and (C) KSR. Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione.
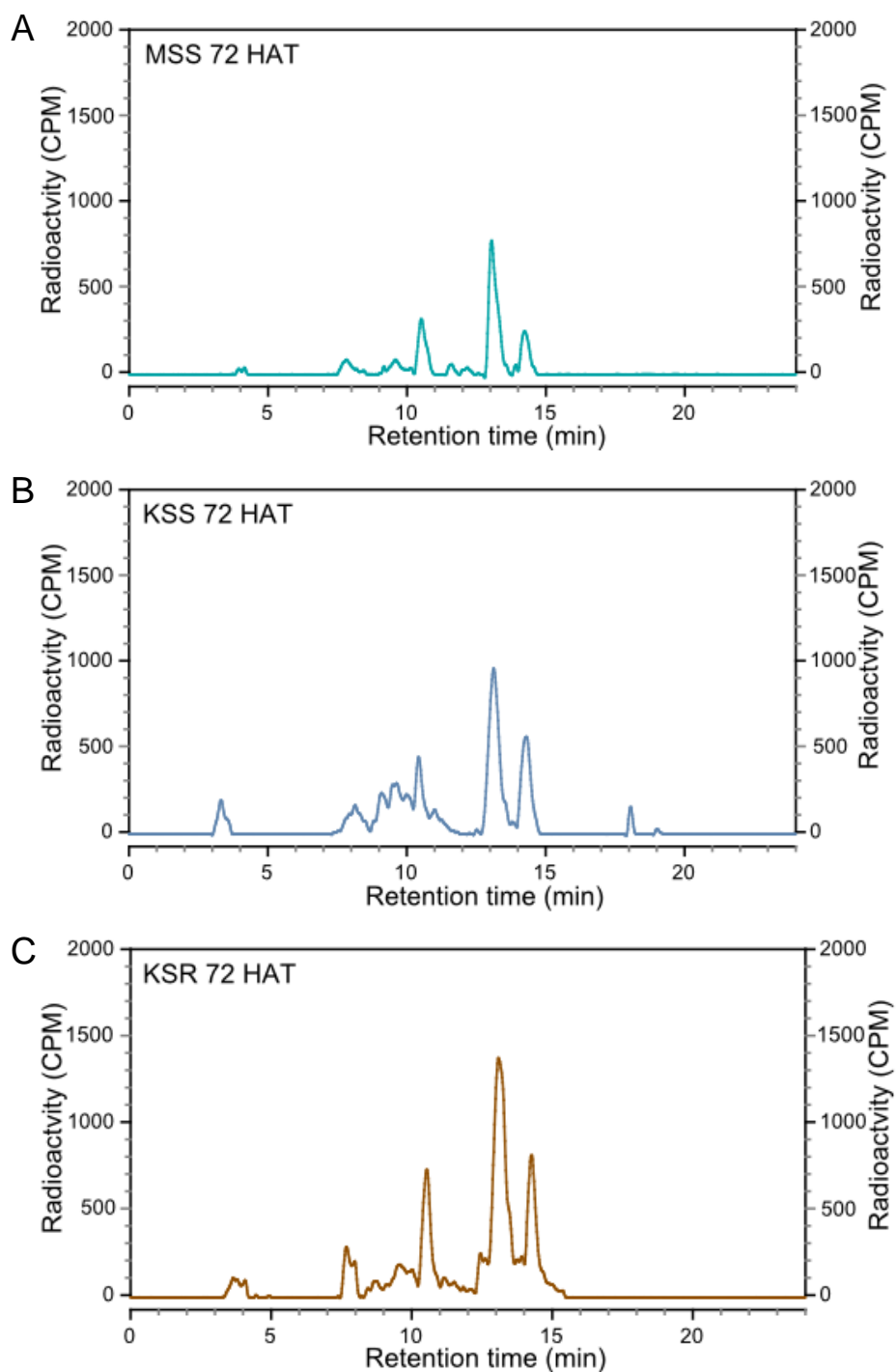
Figure 2.6. Metabolism of [^{14}C] mesotrione in resistant and susceptible Palmer amaranth populations harvested at 72 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [^{14}C] mesotrione and harvested. (A) MSS (B) KSS and (C) KSR. Peak retention time around 18.1 min is the mesotrione (input) and other peaks 13.1 and 14.3 min are the major metabolites of [^{14}C] mesotrione.
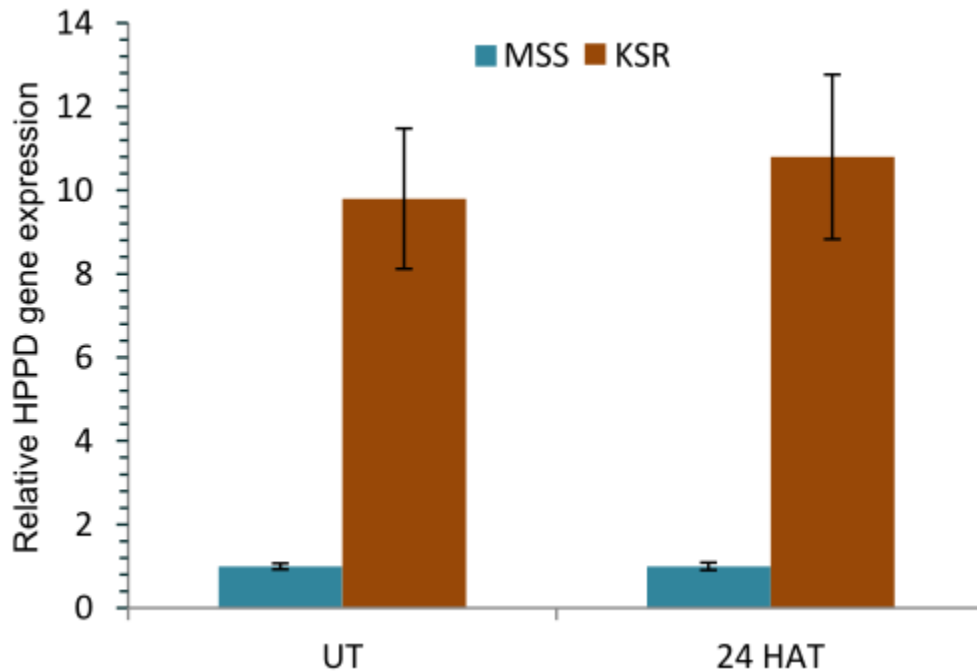
Figure 2.7. The amount of *HPPD* gene expression was normalized to the corresponding level of CPS. The amount of *HPPD* gene expression in the susceptible Palmer amaranth populations was normalized to 1, so that the values of resistant Palmer amaranth populations are presented as the fold-increase relative to each susceptible population. *HPPD* gene expression in MSS and KSR in untreated (UT) Palmer amaranth and 24 HAT with mesotrione. Bars represent the means ± SE.
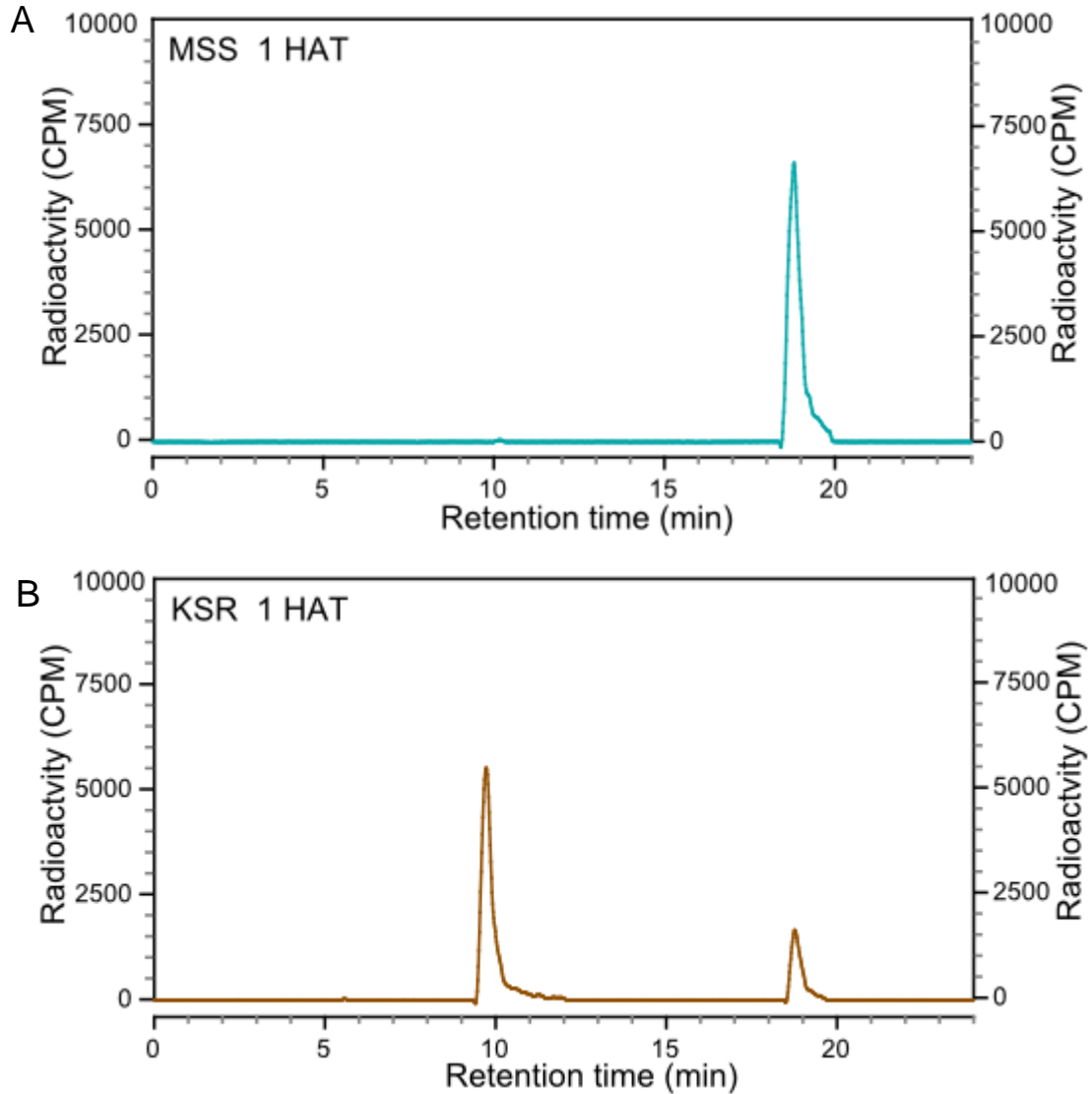
Figure 3.1. Metabolism of [U-^{14}C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 1 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [U-^{14}C] atrazine and analyzed. (A) MSS and (B) KSR. Peak retention time around 18.7 min is the [U-^{14}C] atrazine (input) and other peak at 9.7 min is the major metabolite of [^{14}C] of atrazine, GSH-[^{14}C] of atrazine.
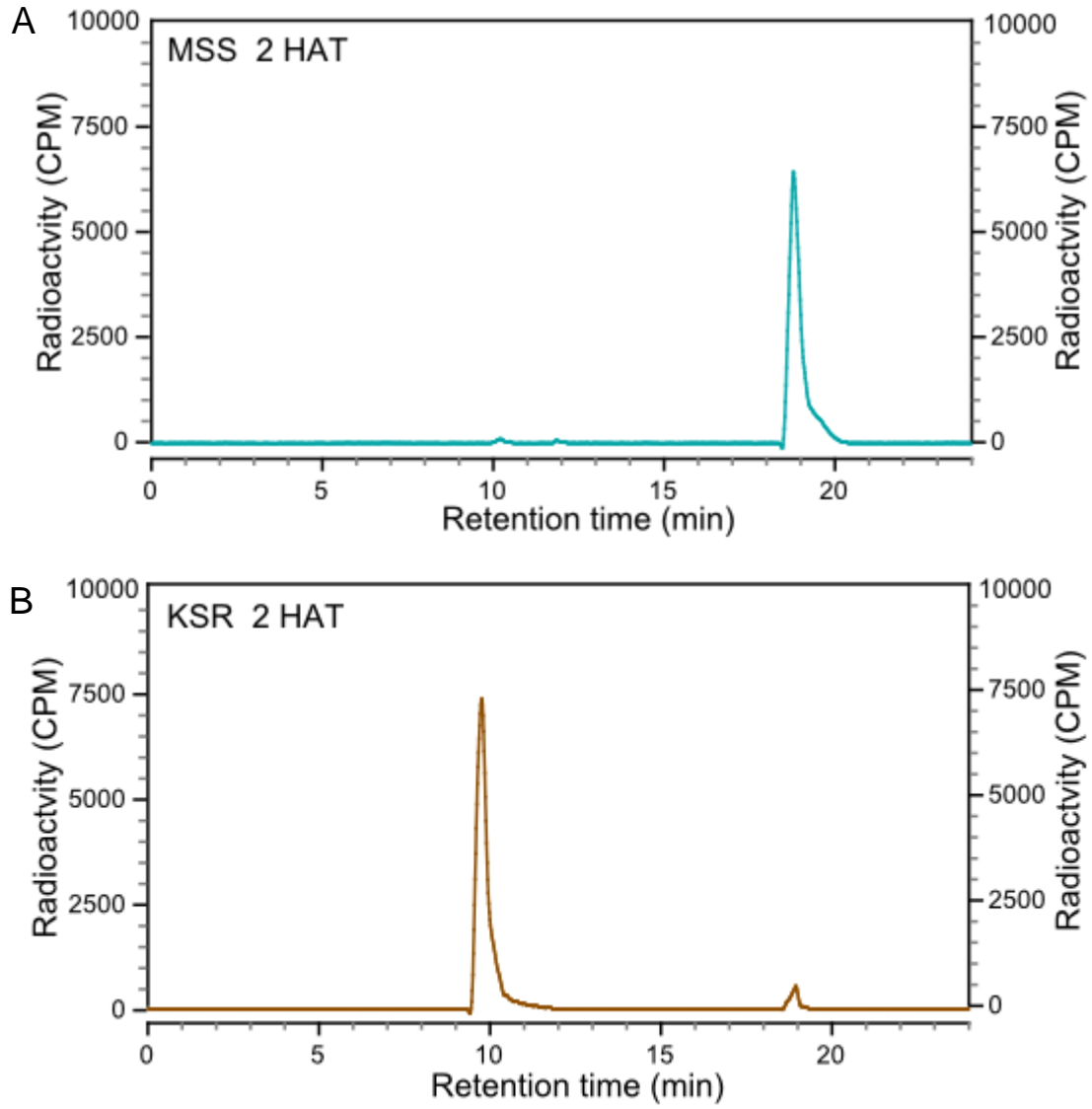
Figure 3.2. Metabolism of [U-^{14}C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 2 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [U-^{14}C] atrazine and analyzed. (A) MSS and (B) KSR. Peak retention time around 18.7 min is the [U-^{14}C] atrazine (input) and other peak at 9.7 min is the major metabolite of [^{14}C] of atrazine, GSH-[^{14}C] of atrazine.

Figure 3.3. Metabolism of [U-^{14}C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 4 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [U-^{14}C] atrazine and analyzed. (A) MSS and (B) KSR. Peak retention time around 18.7 min is the [U-^{14}C] atrazine (input) and other peak at 9.7 min is the major metabolite of [^{14}C] of atrazine, GSH-[^{14}C] of atrazine.
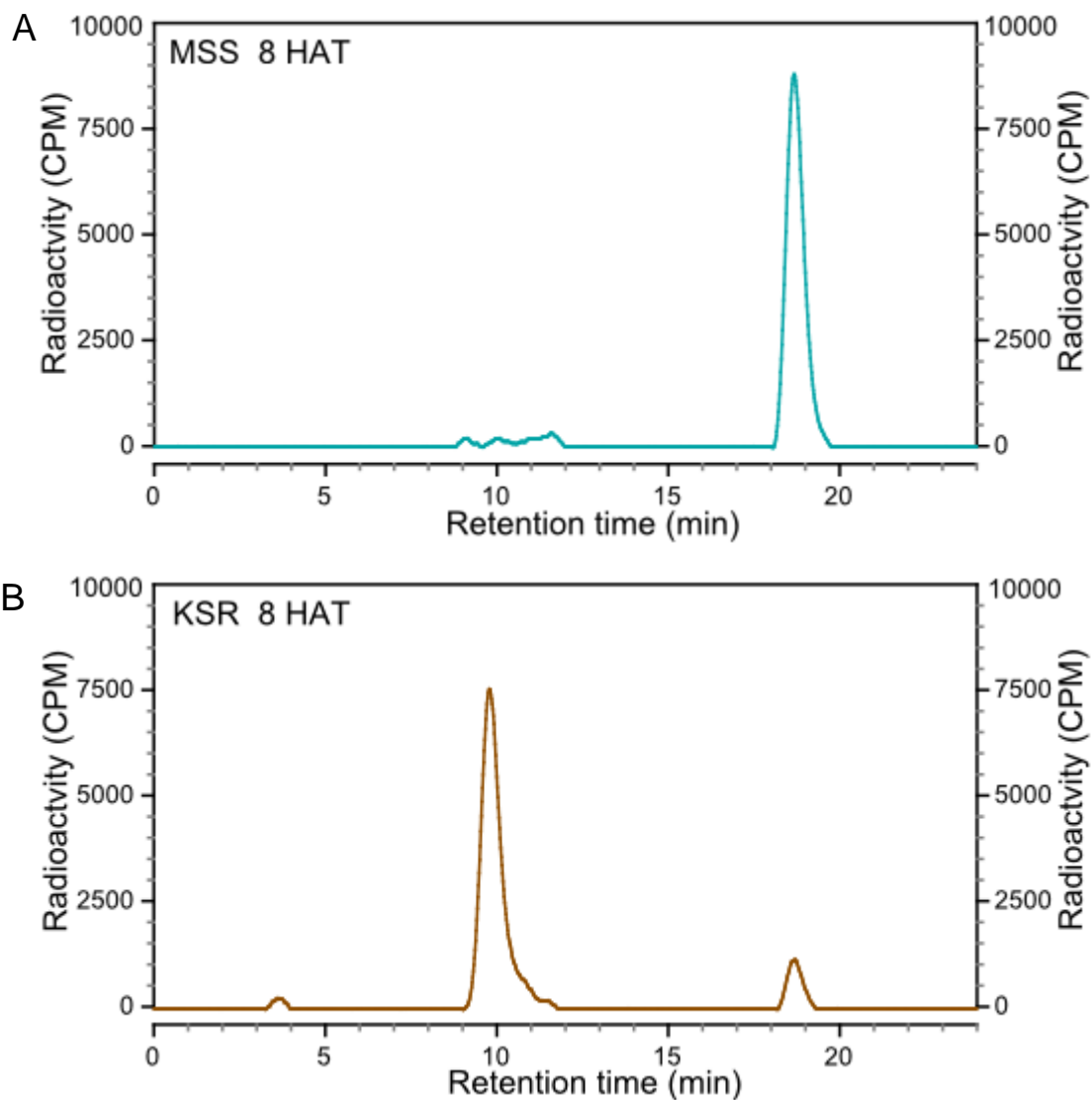
Figure3. 4. Metabolism of [U-^{14}C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 8 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [U-^{14}C] atrazine and analyzed. (A) MSS and (B) KSR. Peak retention time around 18.7 min is the [U-^{14}C] atrazine (input) and other peak at 9.7 min is the major metabolite of [^{14}C] of atrazine, GSH-[^{14}C] of atrazine.
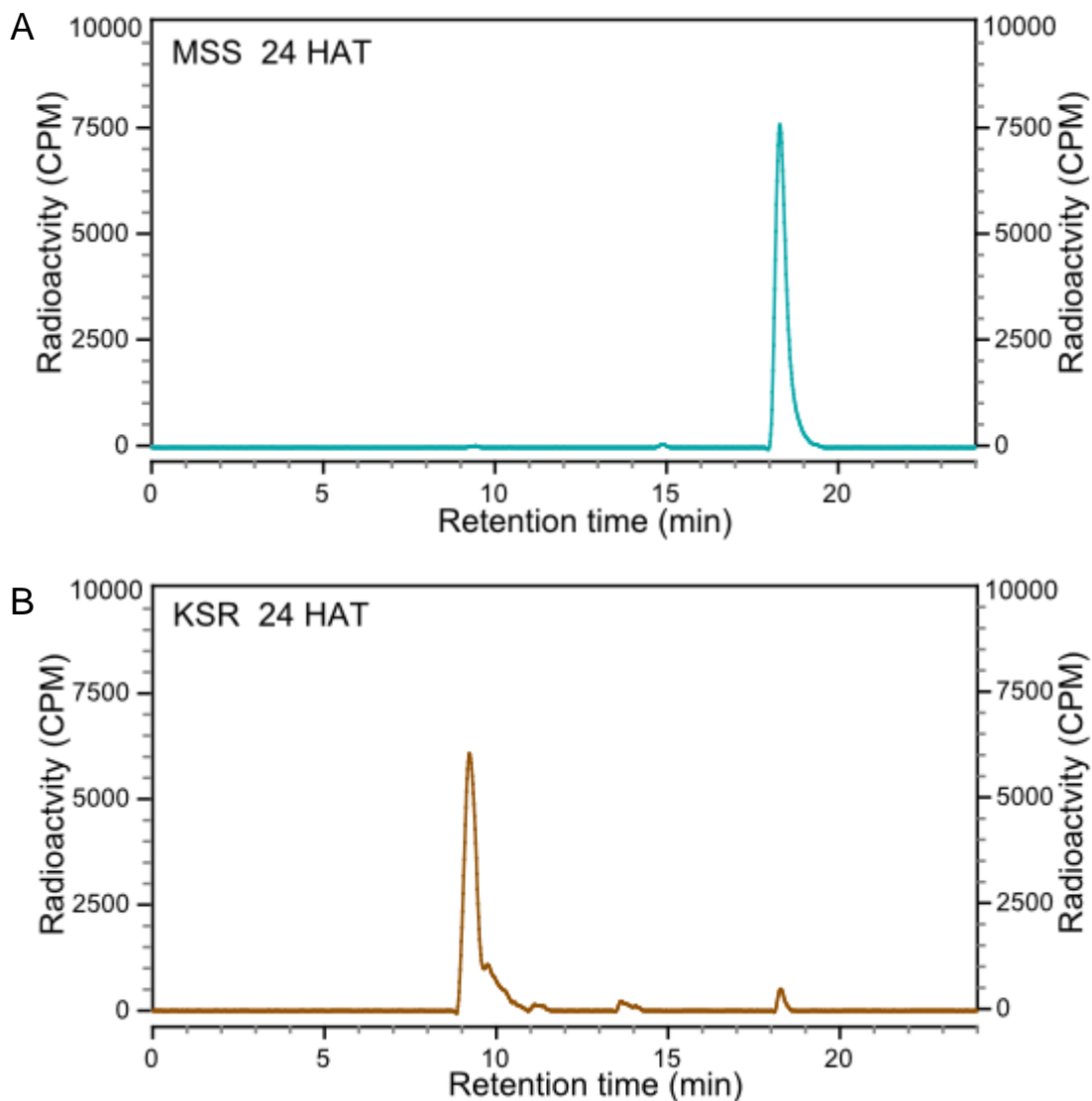
Figure 3.5. Metabolism of [U-^{14}C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 24 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [U-^{14}C] atrazine and analyzed. (A) MSS and (B) KSR. Peak retention time around 18.7 min is the [U-^{14}C] atrazine (input) and other peak at 9.7 min is the major metabolite of [^{14}C] of atrazine, GSH-[^{14}C] of atrazine.
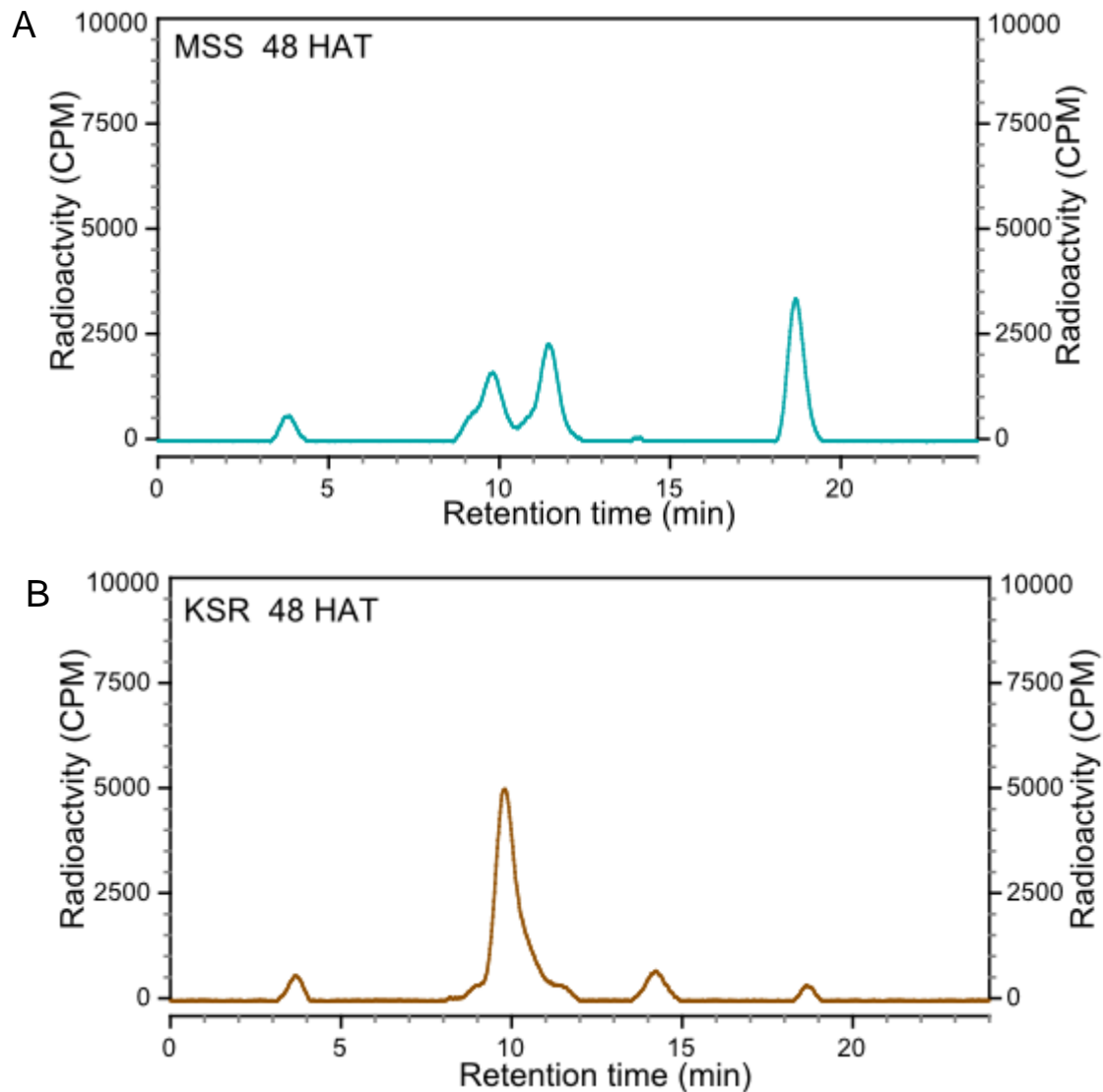
Figure 3.6. Metabolism of [U-^{14}C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 48 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [U-^{14}C] atrazine and analyzed. (A) MSS and (B) KSR. Peak retention time around 18.7 min is the [U-^{14}C] atrazine (input) and other peak at 9.7 min is the major metabolite of [^{14}C] of atrazine, GSH-[^{14}C] of atrazine.

Figure 3. 7. Metabolism of [U-^{14}C] atrazine in susceptible and resistant Palmer amaranth populations harvested at 72 hours after treatment (HAT). Reverse-phase HPLC chromatograms of plants treated with [U-^{14}C] atrazine and analyzed. (A) MSS and (B) KSR. Peak retention time around 18.7 min is the [U-^{14}C] atrazine (input) and other peak at 9.7 min is the major metabolite of [^{14}C] of atrazine, GSH-[^{14}C] of atrazine.
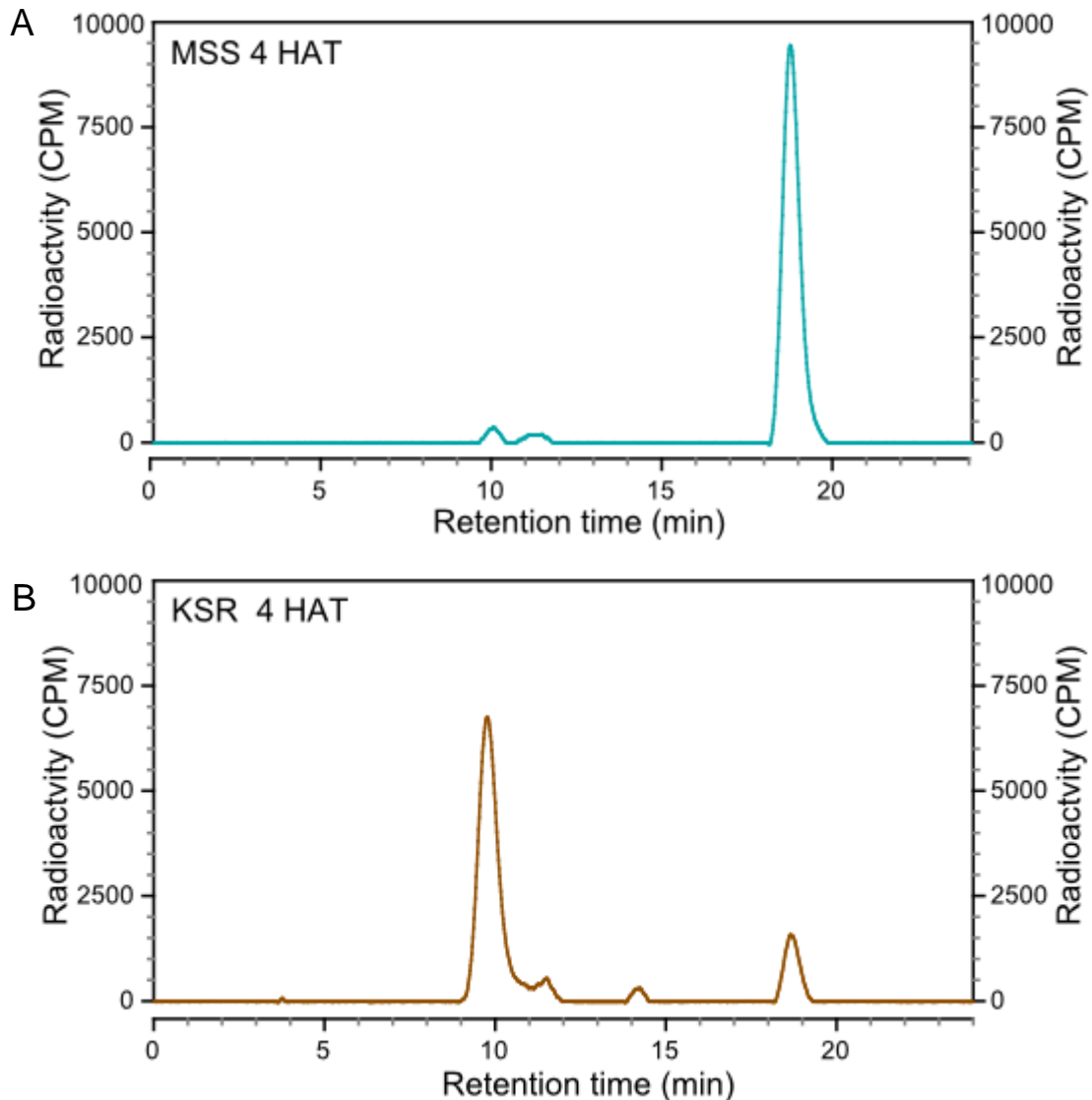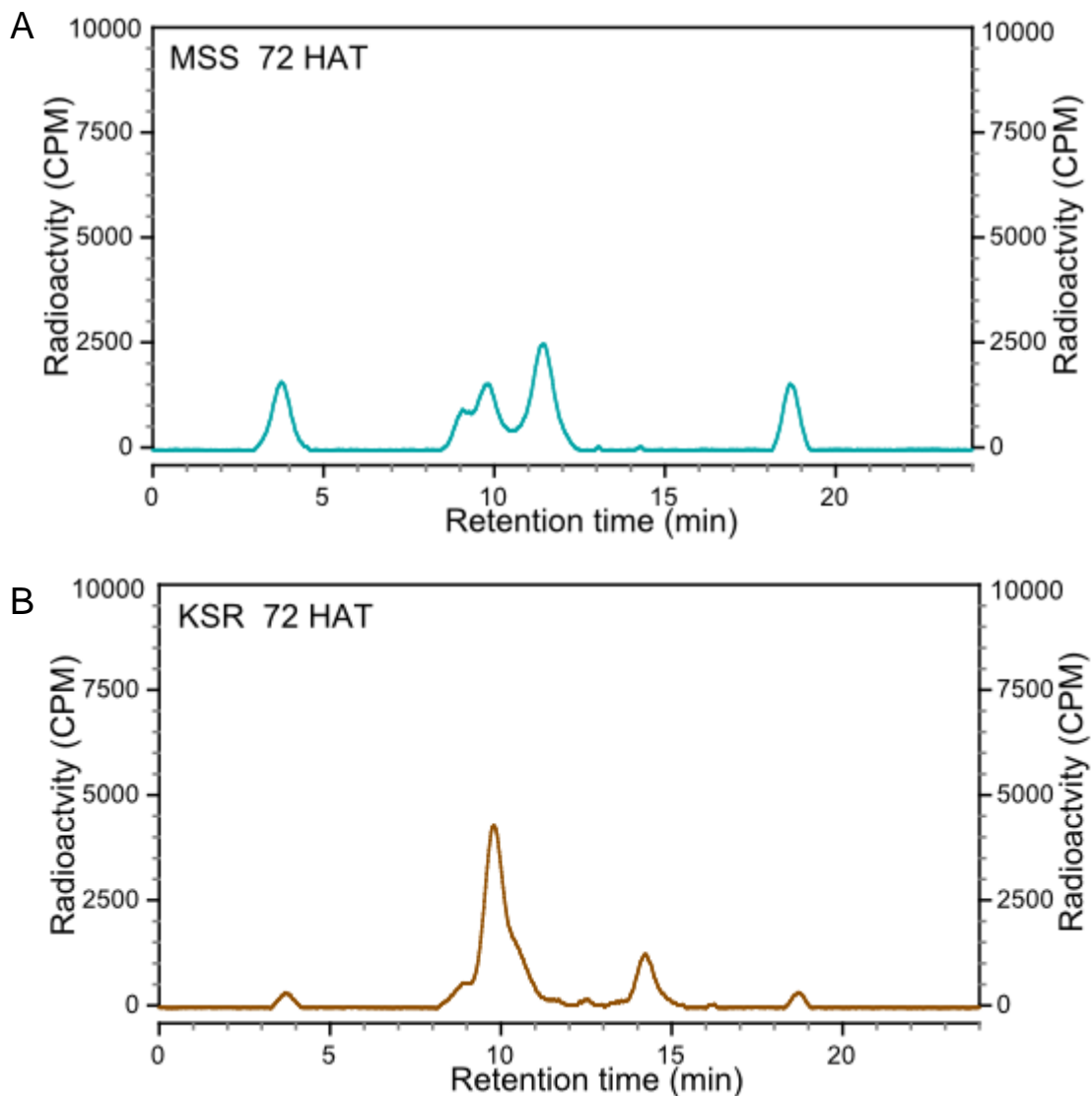
Appendix C - *ALS* and *psbA* gene sequences

Acetolactate synthase (*ALS*) gene sequence:

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                  1                                                                                               130
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       KSR
       MSS

                  131                                                                                             260
Amaranthus  TCCTCGCCGC CCTCTTCAAA TCTCATCATC TTCTTCTACA ATCACCTAAA CCTAAACCTC CTTCCGCTAC TATAACTCAA TCACCTTCGT CTCTCACCGA TGATAAACCC TCTTCTTTTG TTTCCCGATT
       KSR                                              CCTAAACCTC CTTCCGCTAC TATAACTCAA TCACCTTCAT CTCTCACCGA TGATAAACCC TCTTCTTTTG TTTCCCGATT
       MSS                                              CCTAAACCTC CTTCCGCTAC TATAACTCAA TCACCTTCAT CTCTCACCGA TGATAAACCC TCTTCTTTTG TTTCCCGATT

                  261                                                                                             390
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       KSR  TAGCCCTGAA GAACCCAGAA AAGGTTGCGA TGTTCTCGTT GAAGCTCTTG AACGTGAAGG TGTTACCGAT GTTTTTGCTT ACCCTGGTGG AGCTTCCATG GAAATCCATC AAGCTCTTAC TCGTTCTAAT
       MSS  TAGCCCTGAA GAACCCAGAA AAGGTTGCGA TGTTCTCGTT GAAGCTCTTG AACGTGAAGG TGTTACCGAT GTTTTTGCTT ACCCTGGTGG AGCTTCCATG GAAATCCATC AAGCTCTTAC TCGTTCTAAT

                  391                                                                                             520
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       MSS  ATCATTAGAA ATGTTCTTCC TCGACATGAA CAAGGTGGGG TTTTCGCTGC TCAAGGCTAC GCTCGTGCTA CTGGACGGGT TGGAGTTTGT ATTGCCACTT CTGGTCCAGG TGCTACTAAT CTTGTTTCGG

                  521                          Proline CCT/CCC to Serine(TCC)                                    650
Amaranthus  GTTTTGCTGA TGCACTTCTT GACTCAGTCC CGCTTGTCGC CATTACTGGG CAAGTTCCTC GGCGTATGAT TGGTACTGAT GCTTTTCAAG AGACACCTAT AGTTGAGGTA ACACGATCAA TTACTAAGCA
       KSR  GTTTTGCTGA TGCACTTCTT GACTCAGTCC CGCTTGTCGC CATTACTGGG CAAGTTCCTC GGCGTATGAT TGGTACTGAT GCTTTTCAAG AGACTCCTAT TGTTGAGGTA ACTCGATCTA TTACTAAGCA
       MSS  GTTTTGCTGA TGCACTTCTT GACTCAGTCC CGCTTGTCGC CATTACTGGG CAAGTTCCTC GGCGTATGAT TGGTACTGAT GCTTTTCAAG AGACTCCTAT TGTTGAGGTA ACTCGATCTA TTACTAAGCA

                  651                                                                                             780
Amaranthus  TAATTATTTG GTGTTAGATG TTGAGGATAT CCCTAGAATT GTTAAGGAAG CTTTCTTTTT AGCTAATTCT GGTAGACCTG ACCTGTTTT GATTGATATT CCTAAAGATA TTCAGCAACA ATTGGTTGTT
       KSR  TAATTATTTG GTGTTAGATG TTGAGGATAT TCCTAGAATT GTTAAGGAAG CTTTCTTTTT AGCTAATTCT GGTAGACCTG ACCTGTTTT GATTGATATT CCTAAAGATA TTCAGCAACA ATTCGTTGTT
       MSS  TAATTATTTG GTGTTAGATG TTGAGGATAT TCCTAGAATT GTTAAGGAAG CTTTCTTTTT AGCTAATTCT GGTAGACCTG ACCTGTTTT GATTGATATT CCTAAAGATA TTCAGCAACA ATTCGTTGTT

                  781                                                                                             910
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       KSR  CCTAATTGGG AACAGCCCAT TAAATTGGGT GGGTATCTTT CTAGGTTGCC TAAACCCACT TTTTCTGCTA ATGAAGAGGG ACTTCTTGAT CAAATTGTAA GGTTGGTGGG TGAGTCTAAG AGACCTGTGC
       MSS  CCTAATTGGG AACAGCCCAT TAAATTGGGT GGGTATCTTT CTAGGTTGCC TAAACCCACT TTTTCTGCTA ATGAAGAGGG ACTTCTTGAT CAAATTGTAA GGTTGGTGGG TGAGTCTAAG AGACCTGTGC

                  911                                                                                             1040
Amaranthus  TGTATACTGG AGGTGGGTGT TTGAATTCTA GTGAAGAATT AAGGAAATTT GTCGAGTTGA CAGGGATTCC CGTTGCTAGT ACTTTAATGG GGTTGGGGGC TTTCCCTTGT ACTGATGATT TATCACTTCA
       KSR  TGTATACTGG AGGTGGGTGT TTGAATTCTA GTGAAGAATT AAGGAAATTT GTCGAATTGA CAGGGATTCC CGTTGCTAGT ACTTTAATGG GGTTGGGGGC TTTCCCTTGT ACTGATGATT TATCACTTCA
       MSS  TGTATACTGG AGGTGGGTGT TTGAATTCTA GTGAAGAATT AAGGAAATTT GTCGAATTGA CAGGGATTCC CGTTGCTAGT ACTTTAATGG GGTTGGGGGC TTTCCCTTGT ACTGATGATT TATCACTTCA

                  1041                                                                                            1170
Amaranthus  AATGTTGGGA ATGCACGGGA CTGTGTACGC GAATTACGCG GTGGATAAGG CTGATTTGTT GCTTGCTTTC GGGGTTAGGT TTGATGATCG AGTGACTGGG AAGCTCGAGG CGTTTGCTAG CCGGGCTAAG
       KSR  AATGTTGGGA ATGCACGGGA CTGTGTAGGC GAATTACGCG GTTGATAAGG CTGATTTGTT GCTTGCTTTC GGGGTTAGGT TTGATGATCG AGTGACTGGG AAGCTCGAGG CGTTTGCTAG CCGGGCTAAG
       MSS  AATGTTGGGA ATGCACGGGA CTGTGTAGGC GAATTACGCG GTTGATAAGG CTGATTTGTT GCTTGCTTTC GGGGTTAGGT TTGATGATCG AGTGACTGGG AAGCTCGAGG CGTTTGCTAG CCGGGCTAAG

                  1171                                                                                            1300
Amaranthus  ATTGTGCACA TCGATATCGA TTCTGCTGAA ATCGGGAAGA ATAAGCAACC TCATGTGTCG ATTTGTGGTG AAATTAAAGT TGCATTACAG GGTTTGAATA AGATTTTGGA ATCTAGAAAA GGAAAGCTGA
       KSR  ATTGTGCACA TTGACATCGA TTCTGCTGAA ATCGGGAAGA ATAAGCAACC TCATGTGTCG ATTTGTGGTG ATGTTAAAGT GGCATTACAG GGTTTGAATA AGATTTTGGA ATCTAGAAAA GGAAAGCTGA
       MSS  ATTGTGCACA TTGACATCGA TTCTGCTGAA ATCGGGAAGA ATAAGCAACC TCATGTGTCG ATTTGTGGTG ATGTTAAAGT GGCATTACAG GGTTTGAATA AGATTTTGGA ATCTAGAAAA GGAAAGCTGA

                  1301                                                                                            1430
Amaranthus  AATTGGATTT CTCTAATTGG AGGGAGGAGT TGAATGAGCA GAAAAAGAAG TTTCCTTTGA GTTTTAAGTC TTTCGGGGAC GCAATTCCTC CGCAATATGC CATTCAGGTT CTGGACGAGT TGACGTGGG
       KSR  AATTGGATTT CTCTAATTGG AGGGAGGAGT TGAATGAGCA GAAAAAGAAG TTTCCTTTGA GTTTTAAGAC TTTCGGGGAC GCAATTCCTC CGCAATACGC CATTCAGGTT CTTCACGAGT TGACGAAGGG
       MSS  AATTGGATTT CTCTAATTGG AGGGAGGAGT TGAATGAGCA GAAAAAGAAG TTTCCTTTGA GTTTTAAGAC TTTCGGGGAC GCAATTCCTC CGCAATACGC CATTCAGGTT CTTCACGAGT TGACGAAGGG

                  1431                                                                                            1560
Amaranthus  TGATGCGATT GTAAGTACCG GTGTTGGGCA GCACCAAATG TGGGCTGCCC AATTCTATAA GTACCGAAAT CCTCGCCAAT GGCTGACCTC GGGTGGTTTG GGGGCTATGG GGTTTGGTCT ACCAGCCTGC
       KSR  TGATGCGGTT GTAAGTACCG GTGTTGGGCA GCACCAAATG TGGGCTGCCC AATTCTATAA GTACCGAAAT CCTCGCCAAT GGCTGACCTC GGGTGGTTTG GGGGCTATGG GGTTTGGTCT ACCAGCCTGC
       MSS  TGATGCGGTT GTAAGTACCG GTGTTGGGCA GCACCAAATG TGGGCTGCCC AATTCTATAA GTACCGAAAT CCTCGCCAAT GGCTGACCTC GGGTGGTTTG GGGGCTATGG GGTTTGGTCT ACCAGCCTGC

                  1561                                                                                            1690
Amaranthus  TATGGAGCTG CTGTTGCTCG ACCAGATGCG GTGGTTGTAG ACATTGATGG GGATGGGAGT TTTATCATGA ATGTTCAAGA GTTGGCTACG ATTAGGGTAG AGAATCTCCC GGTTAAAATC ATGCTCTTGA
       KSR  TATGGAGCTG CTGTTGCTCG ACCAGATGCG GTGGTTGTAG ACATTGATGG GGATGGGAGT TTTATCATGA ATGTTCAAGA GTTGGCTACG ATTAGGGTTG AGAATCTCCC GGTTAAAATC ATGCTCTTGA
       MSS  TATGGAGCTG CTGTTGCTCG ACCAGATGCG GTGGTTGTAG ACATTGATGG GGATGGGAGT TTTATCATGA ATGTTCAAGA GTTGGCTACG ATTAGGGTTG AGAATCTCCC GGTTAAAATC ATGCTCTTGA

                  1691                                                                                            1820
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       KSR  ACAATCAACA TTTAGGTATG GTTGTTCAAT TGGAAGATCG ATTTTACAAA GCTAACCGGG CACATACATA CCTCGGGAAT CCTTCCAATT CTTCCGAAAT CTTCCCGGAT ATGCTTAAAT TTGCTGAAGC
       MSS  ACAATCAACA TTTAGGTATG GTTGTTCAAT TGGAAGATCG ATTTTACAAA GCTAACCGGG CACATACATA CCTCGGGAAT CCTTCCAATT CTTCCGAAAT CTTCCCGGAT ATGCTTAAAT TTGCTGAAGC

                  1821                                                                                            1950
Amaranthus  ATGTGATATA CCAGCAGCCC GTGTTACCAA GGTGAGCGAT TTAAGGGCTG CAATTCAAAC AATGTTGGAT ACGCCAGGAC CATATCTGCT GGATGTAATC GTACCACATC AGGAGCATGT GCTGCCTATG
       KSR  ATGTGATATA CCAGCAGCTC GTGTTACCAA GGTGAGCGAT TTAAGGGCTG CAATTCAAAC AATGTTGGAT ACTCCAGGAC GTATCTGCT GGATGTAATC GTACCACATC AGGAGCATGT GCTGCCTATG
       MSS  ATGTGATATA CCAGCAGCTC GTGTTACCAA GGTGAGCGAT TTAAGGGCTG CAATTCAAAC AATGTTGGAT ACTCCAGGAC GTATCTGCT GGATGTAATC GTACCACATC AGGAGCATGT GCTGCCTATG

                  1951              2013
Amaranthus  ATCCCTAGCG GTGCCGCCTT CAAGGACACC ATCACAGAGG GTGATGGAAG AAGGGCTTAT TAG
       KSR  ATCCCTAGCG GTGCCGCCTT CAAGGACACC ATCACAGAGG GTGATGGAAG AAGGGCTTAT TAG
       MSS  ATCCCTAGCG GTGCCGCCTT CAAGGACACC ATCACAGAGG GTGATGGAAG AAGGGCTTAT TAG
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Figure 1. Acetolactate synthase (*ALS*) gene sequence of resistant (KSR) and susceptible (MSS) *Amaranthus Palmeri* in comparison to *Amaranthus sp* complete coding sequence (Accession No. U55852). The nucleotides highlighted in green represent the polymorphism between the species and codon represented in blue codes for serine indicating a mutation at proline197 position. The aminoacid position is in reference to *Arabidopsis* ALS sequence.

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psbA gene sequence:

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MSS .......... .......... .......... TTCTGATGGT ATGCCTCTAG GAATCTCTGG TACTTTCAAC TTTATGATCG TATTCCAGGC TGAGCACAAC ATCCTTATGC ACCCATTTCA CATGTTAGGT
KSR .......... .......... .......... TTCTGATGGT ATGCCTCTAG GAATCTCTGG TACTTTCAAC TTTATGATCG TATTCCAGGC TGAGCACAAC ATCCTTATGC ACCCATTTCA CATGTTAGGT

MSS GTAGCTGGTG TATTCGGCGG CTCCCTATTT AGTGCTATGC ATGGTTCCTT GGTAACTTCT AGTTTGATCA GGGAAACCAC AGAAAATGAA TCTGCTAACG AAGGTTACAG ATTCGGTCAA GAGGAAGAAA
KSR GTAGCTGGTG TATTCGGCGG CTCCCTATTT AGTGCTATGC ATGGTTCCTT GGTAACTTCT AGTTTGATCA GGGAAACCAC AGAAAATGAA TCTGCTAACG AAGGTTACAG ATTCGGTCAA GAGGAAGAAA
                                                                         Serine264
MSS CTTATAACAT CGTAGCTGCT CACGGTTATT TTGGTCGATT GATCTTCCAA TATGCTAGTT TCAACAACTC TCGTTCTTTA CACTTCTTCT TAGCTGCTTG GCCTGTAATC GGTATTTGGT TTACTGCTTT
KSR CTTATAACAT CGTAGCTGCT CACGGTTATT TTGGTCGATT GATCTTCCAA TATGCTAGTT TCAACAACTC TCGTTCTTTA CACTTCTTCT TAGCTGCTTG GCCTGTAATC GGTATTTGGT TTACTGCTTT

MSS GGGTATTAGT ACTATGGCTT TCAACCTAAA CGGTTTCAAC TTCAACCAAT CTGTAGTTGA TAGTCAAGGT CGTGTAATTA ACACCTGGGC TGATATCATT AACCGTGCTA ACCTTGGTAT GGAAGTTATG
KSR GGGTATTAGT ACTATGGCTT TCAACCTAAA CGGTTTCAAC TTCAACCAAT CTGTAGTTGA TAGTCAAGGT CGTGTAATTA ACACCTGGGC TGATATCATT AACCGTGCTA ACCTTGGTAT GGAAGTTATG

MSS CATGAACGTA .......... ..........
KSR CATGAACGTA .......... ..........
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Figure 2. Nucleotide sequence of portion of *psbA* gene sequenced from resistant (KSR) and susceptible (MSS) *Amaranthus Palmeri.* The codon highlighted in blue codes for serine indicating no mutation at 264 position between KSR and MSS. The aminoacid position is in reference to *Arabidopsis psbA* protein sequence.