FROM THE SIMPLE TO THE COMPLEX: AN EXAMINATION OF DIFFERENT HERBICIDE RESISTANCES

BY

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THESIS

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ABSTRACT

Herbicide resistance is the result of an evolutionary adaptation that some agronomic weed species have obtained from intense human-driven selection. The mechanisms by which these plants resist herbicides can be diverse. For example, self-pollinating species, such as goosegrass (*Eleusine indica*), frequently have target-site resistance mechanisms controlled by a single gene. Outcrossing species, such as waterhemp (*Amaranthus tuberculatus*), in addition to having herbicide resistance conferred by a target-site mutation controlled by a single gene, often evolve non-target-site-based resistance mechanisms controlled by multiple genes. The overall purpose of this thesis was to determine the genetics and inheritance of both target-site- and non-target-based resistances to provide beneficial insights into resistance evolution, adaptation dynamics and management practices. Chapter 2 discusses research conducted to determine if a Tennessee glyphosate-resistant (TennGR) goosegrass population had target-site resistance (TSR) that previously had been associated with glyphosate resistance in other populations [specifically a Pro106Ser substitution in the 5-enolypyruvyl-shikimate-3-phosphate synthase (EPSPS) gene]. Sequencing of the entire TennGR goosegrass EPSPS gene was performed and compared to that of the sensitive Tennessee population (TennGS). The results indicated that the population did contain the anticipated Pro106Ser mutation. An F_2 population was derived and used in a whole-plant dose-response experiment to compare the three segregating EPSPS genotypes. This experiment revealed that the Pro106Ser mutation was the sole mechanism of glyphosate resistance in the TennGR population. Chapter 3 discusses research conducted to determine the inheritance of two distinct non-target-site resistances, to atrazine and mesotrione, in a population of waterhemp (MCR). Crosses were performed to

generate F_1, backcross (BC), and F_2 lines. Through separate atrazine and mesotrione dose responses experiments, it was determined that the responses of reciprocal F_1 lines did not differ and were intermediate to that of the R and S parental populations, indicating resistance for both herbicides was nuclear inherited. Segregation analysis in F_2 and BC_S lines indicated inheritance was controlled by a single gene for atrazine resistance and multiple genes for mesotrione resistance. The fourth and final chapter provides concluding remarks and future research opportunities.

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CHAPTER 1

Introduction

1.1 Herbicide Resistance in Weeds

Weeds are unwanted or misplaced plants that have been agronomical pests since the beginning of agriculture (Powles and Yu 2010). Yield loss, competition for water, and reduction in crop quality are a few examples of how weeds can adversely impact crops. In broadacre crops, cost-effective weed management almost always involves the use of herbicides. In particular, since the introduction of genetically engineered crops, the use of an integrated pest management system has largely been replaced by the sole use of herbicides. With heavy reliance on herbicides, weeds quickly adapted and evolved resistance. Today, there are currently 239 plant species (139 dicots and 100 monocots) that are resistant to herbicides encompassing 22 of the 25 known sites of action (Heap 2015). Resistant weeds are less sensitive to herbicides by a variety of mechanisms that can be divided into two broad categories: target-site resistance (TSR) and non-target-site resistance (NTSR) (Délye et al. 2013).

1.1.1 Target-Site Resistance

Most herbicides target specific enzymes or proteins. Resistance typically occurs when an amino acid within the target site is changed resulting in decreased binding affinity of the herbicide. A fitness cost could result if a mutation endowing resistance impairs the enzyme functionality and/or the performance of the plant (Vila-Aiub et al. 2009). Numerous cases of

herbicide resistance conferred by a point mutation have been documented. For example, Tranel et al. (2015) have cataloged numerous point mutations in the acetolactate synthase (ALS) gene that confer resistance in various weed species. A unique incidence worth mentioning belongs to a population of waterhemp with a target-site-based mechanism that confers resistant to protoporphyrinogen oxidase (PPO) inhibitors; here resistance involves a codon deletion rather than a single nucleotide substitution (Patzoldt et al. 2006). A third type of target-site-based resistance is the overproduction of the herbicide-binding protein (Preston and Mallory-Smith 2001). Amplification of the EPSPS gene was initially discovered in Palmer amaranth (*Amaranthus palmeri*); resistant plants contained between 5- and 160-fold more copies of the target-site gene compared to sensitive plants (Gaines et al. 2010). By producing excessive amounts of EPSPS, resistant plants create extra EPSPS protein available for the herbicide and the natural substrate. Hence, the surplus EPSPS enzymes function as a "molecular sponge", soaking up glyphosate and permitting normal functioning of the shikimic acid pathway (Powles 2010).

Resistant plants are often categorized as either having a high-level of resistance (plants surviving more than 10 times the standard herbicide rate) or having a low level resistance (not able to survive more than a few times the standard use rate). High-level resistance has been documented in the following classes of herbicides: Acetyl-coenzyme A carboxylase (ACCase) inhibitors, ALS inhibitors, and photosystem II (PSII) inhibitors (Pfister and Arntzen 1979; Devine and Shukla 2000; Preston et al. 2006). Both ALS and ACCase have many different resistance-endowing gene mutations, and the level of resistance tends to vary among the different mutation (Powles and Yu 2010). Examples of low-level resistance due to target-site alterations

include goosegrass and some populations of annual ryegrass (*Lolium rigidum)* resistant to glyphosate (Baerson et al. 2002; Perez and Kogan 2003). Low levels of resistance to glyphosate are most often conferred by a Pro106 substitution. Pro106 substitutions confer only a modest degree of glyphosate resistance because glyphosate is a competitive inhibitor of the second substrate, phosphoenolpyruvate (PEP) (Boocock and Coggins 1983), and is believed to be a transition state mimic of the catalyzed reaction course (Schönbrunn et al. 2001).

It is relatively easy to study the molecular mechanisms of TSR (Yuan et al. 2007); for instance, molecular markers are usually available or could be quickly developed with minimum research. Evidence can be found for TSR in most of the major groups of herbicides marketed today. In many cases, TSR has arisen spontaneously following repeated doses of herbicides or families of herbicides in a cropping system (Devine and Shukla 2000).

1.1.2 Non-Target-Site Resistance

NTSR results from alterations in non-target sites that endow reduced herbicide uptake/translocation (including herbicide sequestration), increased rates of herbicide detoxification, or decreased rates of herbicide activation (Powles and Yu 2010). Stated by Délye (2013), NTSR is part of a plant's stress response. The plant employs a dynamic process of 'protectors' that directly interfere with the herbicide access to the target site, along with regulators controlling the 'protectors' expression (Délye 2013). Research with NTSR is often complicated because the genetics involved with plants' response to stress is often under the control of many different genes. NTSR can even confer unpredictable cross-resistance to herbicides with diverse sites of action, including herbicides not currently marketed (Petit et al.

2010a, 2010b). In a ryegrass population (VLR69) NTSR has been documented to 16 different active ingredients (with a total of 9 different sites of action) with inheritance being both monogenic and polygenic, depending on the herbicide (Preston 2003; Busi et al. 2011, 2013). Also, populations of black-grass (*Alopecurus myosuroides)* have been documented to be resistant to all the preferred herbicides listed for black-grass control in wheat (*Triticum* spp.) as a result of NTSR (Délye et al. 2011). NTSR is now considered the predominant type of resistance in two of the most commonly used herbicides; glyphosate and acetyl-CoA carboxylase inhibitors (Délye 2013). NTSR is the sole mechanism of resistance for the following herbicide groups: photosystem I inhibitors, inhibitors of fatty acid elongase (VLCFA) and plant growth regulators (PGRs) that stimulate the transport inhibitor response protein 1 (Délye et al. 2013). Because not all mechanisms of NTSR are clearly known or understood, applying a herbicide to a field that has a history of NTSR could risk the chance of herbicide resistance to that herbicide because of possible upregulation of NTSR mechanisms (Neve and Powles 2005; Délye et al. 2011; Guo et al. 2015; Busi et al. 2013).

1.1.3 Genetics and Inheritance of Herbicide Resistances

Inheritance of herbicide resistance is either determined by nuclear inheritance or cytoplasmic inheritance. Most herbicide resistances examined thus far are governed by a single, nuclear-encoded gene with partial or full dominance. A resistance trait that has full or partial dominance spreads throughout the population much faster when compared with resistance conferred by a recessive trait. For nuclear inheritance, resistance alleles are carried by both male and female gametes; consequently, both pollen and seed disseminate the resistance.

Cytoplasmic inheritance is solely documented in triazine-resistant weeds and, since only female plants transmit the resistance alleles, the rate at which resistance spreads is drastically slower (Jasieniuk et al. 1996; Gaur and Sharma 2013).

Although the appearance of herbicide-resistant weed populations has been well documented, information on the inheritance of resistance, particularly for NTSR (Lorraine-Colwill et al. 2001), is limited to a few well-characterized resistant weed populations (Letouzé and Gasquez 2001; Preston 2003; Busi et al. 2011, 2013). Control of NTSR pathways in herbicide sensing and herbicide stress-response activation is largely unknown. Proteins from several different families have been identified in resistant weeds that are relevant to herbicide degradation (glutathione-*S*-transferases, cytochrome P450s, glycosyl-transferases, esterases, hydrolases), compartmentalization (transporter proteins) and compensation (oxidases, peroxidases) (Délye et al. 2013). Data describing the genetic basis of NTSR are widely lacking, but progress is being made in identifying the genes involved with evolved metabolic herbicide resistance (Mithila et al. 2012; Cummins et al. 2013; Gaines et al. 2014; Iwakami et al. 2013, 2014; Duhoux et al. 2015). NTSR inheritance can be controlled as a monogenic trait, but frequently many genes are involved (polygenic), and this is especially true with herbicide detoxification by cytochrome P450 monooxygenases (P450). Plant P450s form a large family of proteins that catalyze a diverse array of biosynthetic reactions for lignin, pigments, hormones, UV protectants, fatty acids and defense compounds (Schuler 1996). Crops, such as maize and wheat, have the innate capacity for P450-mediated metabolism of several herbicides (Werck-Reichhart et al. 2000; Siminszky 2006). The current understanding of P450-mediated herbicide metabolism in plants suggests that there are likely many P450 isoforms with varying herbicide

substrate specificities, which would give variable capacity for herbicide metabolism resulting in possible cross-resistance to herbicides with different modes of action (Busi et al. 2011).

NTSR mechanisms commonly have polygenic inheritance, but there have been reports of species with NTSR due to monogenic inheritance. Two distinct velvetleaf (*Abutilon theophrasti*) populations (Gronwald et al. 1989; Gary et al. 1996) have shown increased GST-catalyzed atrazine detoxification, and this resistance was controlled by a single, incompletely dominant gene (Anderson and Gronwald 1987). Another example of NTSR due to monogenic inheritance is paraquat resistance in an annual ryegrass population attributed to reduced herbicide translocation via a single nuclear gene with nearly full dominance (Yu et al. 2009).

As stated previously, TSR is relatively easy to research because inheritance is usually under monogenic control due to dominant or semi-dominant alleles. Complication can arise when TSR is controlled by recessive alleles. There are only a few cases of resistance being conferred by recessive genes: dinitroaniline resistance in green foxtail (*Setaria virdis*) (Jasieniuk et al. 1994), goosegrass (Zeng and Baird 1997), and wild oat (*Avena fatua*) (Kern et al. 2001), and clopyralid and picloram resistance in yellow star-thistle (*Centaurea solstitialis*) (Sabba et al. 2003). To elaborate on the complexity of inheritance of herbicide resistance conferred by recessive genes, consider the TSR conferred in microtubule inhibiting herbicides, which was studied in detail for both goosegrass (Zeng and Baird 1997) and green foxtail (Jasieniuk et al. 1994). These studies independently identified an α-tubulin gene mutation resulting in a Thr239Ile substitution. This mutation in both species governed cross-resistance to select microtubule inhibiting herbicides and to select synthetic auxins (Yamamoto et al. 1998;

Anthony et al. 1998; Délye et al. 2004) and gave negative cross-resistance to carbamate herbicides (Anthony et al. 1999; Délye et al. 2004).

Following Hardy-Weinberg assumptions, the initial increase in frequency of a rare, advantageous, dominant allele is more rapid than that of a rare, advantageous, recessive allele because rare alleles are likely found in a heterozygous state. Thus, herbicide resistance conferred by recessive mutations is not available for positive selection until it reaches a high enough frequency to start appearing in the homozygous state (Andrews 2010). Herbicide resistance conferred by one or more recessive genes is predicted to take significantly longer to evolve unless the targeted species is predominately self-pollinating. The only outcrossing species with recessive herbicide resistance is yellow star-thistle (Sabba et al. 2003), whereas all of the other plant species with this type of resistance are predominately self-pollinating.

1.2 Goosegrass

Goosegrass is a problematic weed that was introduced from Asia to the United States. Goosegrass is a serious problem in cotton (*Gossypium hirsutum*), soybean (*Glycine max*), turf, and a number of vegetable crops (Zeng and Baird 1997). It has been ranked as one of the five most troublesome weeds in the world (Holm et al. 1977). Integrated weed management programs involving pre-plant incorporated (PPI), preemergence (PRE) herbicides, and postemergence (POST) herbicides with residual activity have proved useful for goosegrass control (Mueller et al. 2011). Presently, management systems often consisting only of glyphosate have largely replaced this integrated system (Culpepper and York 1998; Wilcut et al.

2003). Repetitive use of the same site of action, especially at a below-label glyphosate dose, tends to favor herbicide resistance in goosegrass due to accelerated selection pressure.

1.2.1 Goosegrass Biology

Goosegrass is a summer annual, completing its entire life cycle over the course of the summer months, which allows for high competition with crops. Goosegrass uses the C_4 pathway for photosynthesis and consequently has the capacity of a higher photosynthetic rate in the presence of high light and high temperature. Goosegrass is easily misidentified with large crabgrass (*Digitaria sanguinalis*) due to similar emergence patterns, similar-shaped leaves and membranous ligules. Unlike large crabgrass, goosegrass does not have hairs on the upper leaf surface and appears white/silver near the stem base. Goosegrass is self-pollinating and, due to high reproductive capabilities, a single plant is capable of producing over 40,000 seeds (Holm et al. 1977). Due to goosegrass' advantageous biology and current management styles, herbicide resistance has evolved for many different sites of action, and this species continues to be a major problem in cropping systems throughout the world.

1.2.2 Herbicide Resistance in Goosegrass

Goosegrass has evolved resistance to numerous herbicides (Heap 2015), including, ALS inhibitors (Valverde et al. 1993), ACCase inhibitors (Leach et al. 1995), bipyridiliums (Buker et al. 2002), glyphosate (Lee and Ngim 2000), glutamine synthase inhibitors (Jalaludin et al. 2010) and dinitroaniline herbicides (Mudge et al. 1984). Resistance to dinitroaniline herbicides is the most documented for goosegrass. Glyphosate-resistant goosegrass has been identified in numerous

countries and several U.S. states (Heap 2015). In Malaysia, a longer growing season and almost continual use of glyphosate have created an intense selection pressure on populations of goosegrass, resulting in stacking of multiple herbicide resistances (Heap 2015).

1.3 Waterhemp

Waterhemp is one of the ten *Amaranthus* species present across the Great Plains region. Historically, the most predominant *Amaranthus* species were the monoecious species such as smooth pigweed *(Amaranthus hybridus L.)*, but in the 1990s a change in farming practices began to favor waterhemp's germination and growth (Hager et al. 2002). Waterhemp possesses a larger genetic diversity due to its dioecious biology. A heavy reliance on chemical control has caused waterhemp to evolve herbicide resistance.

1.3.1 Waterhemp Biology

Waterhemp is a broadleaf weed species with a rapid growth rate attributable to its C_4 pathway (Steckel 2007). It has wind-pollinated flowers, and a single female plant is capable of producing one million seeds (Steckel et al. 2003). It has prolonged emergence throughout the growing season, with seedlings emerging from May to August (Hartzler et al. 1999). Waterhemp is also able to adapt to a variety of growing conditions and can reach heights of three meters or more (Costea et al. 2005). With many beneficial characteristics in combination with recent management styles, herbicide resistance has evolved to many different sites of action.

1.3.2 Herbicide Resistance in Waterhemp

Waterhemp's ability to evolve resistance is due to having a large genetic diversity. This species has evolved resistance to herbicides spanning six different sites of action: PPO inhibitors, ALS inhibitors, PSII inhibitors, glyphosate, 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors, and plant growth regulators (PGRs) (Heap 2015). In the Midwest and other sections of the U.S. it is common to have stacked herbicide resistances across diverse herbicide families within a single waterhemp population (Bell et al. 2013; Schultz et al. 2015; Heap 2015).

1.4 HPPD Inhibitors, Triazines, and Glyphosate

1.4.1 Mode of Action and Herbicidal Characteristics of HPPD Inhibitors

HPPD-inhibiting herbicides are post- and pre-applied systemic herbicides used to control both grass and broadleaf weeds. HPPD-inhibiting herbicides comprise three chemical classes: the isoxazoles, pyrazolones and triketones (Hirai et al. 2002; van Almsick 2009). These chemicals act as competitive inhibitors for the HPPD enzyme, which is the key enzyme in the biosynthesis of tocopherol and plastoquinone. Tocopherols and carotenoids detoxify reactive oxygen species and scavenge for free radicals in plant tissue (Maeda and DellaPenna 2007; Triantaphylidès and Havaux 2009). Also, carotenoids protect chlorophyll from photooxidation (Cazzonelli and Pogson 2010). The herbicidal activity in susceptible plant species causes characteristic bleaching of emerging foliar tissue following application (Matringe et al. 2005). The discovery of HPPD inhibitors came from the allelopathic properties observed in the bottlebrush plant *Calistemon* spp. (Lee et al. 1997), which produced bleaching symptoms induced by the chemical compound leptospermone (Hellyer 1968). Zeneca Ag Products (later

Syngenta) added a chlorine group to an analog of leptospermone which produced similar bleaching symptom in plants, thus leading to the discovery of the herbicidal triketones (Michaely and Kraatz 1983; Lee et al. 1998; Mitchell et al. 2001).

The selectivity in corn (*Zea mays*) and select grass crops is attributed to reduced herbicide uptake, rapid metabolism and an innately less sensitive target site. When investigating the rapid metabolism of HPPD inhibitors, detoxification by way of cytochrome P450s was speculated. This hypothesis was tested by applying mesotrione to corn that was previously pre-treated with malathion, a known P450 inhibitor, which resulted in corn mortality. Conversely, a sole application of either mesotrione or malathion resulted in plants displaying no visible injury (Mitchell et al. 2001).

1.4.2 Mode of Action and Herbicidal Characteristics of Triazines

PSII inhibitors are post- and pre-applied herbicides used to control grass and broadleaf weeds. Triazines can provide residual control of sensitive dicot weeds for up to sixty days, depending on soil conditions (Krutz et al. 2009). Triazine injury from pre-applied herbicides is typically characterized by chlorosis of the older leaves initiating around the leaf margins followed by necrosis. This type of injury is due to translocation of the herbicide through the xylem only. Triazines inhibit photosynthetic electron transport by displacing plastoquinone from the active site on the D1 protein subunit of PSII (Hess 2000). When the PSII electron transport chain is inhibited, the production of NADPH and ATP is discontinued, which subsequently terminates the carbon reduction cycle leading to carbohydrate starvation and oxidative stress caused by reactive oxygen species (Hess 2000). This method of disrupting PSII is

shared with a few other structurally different chemical families of PSII inhibitors, including triazinones, uracils and phenylureas.

Selectively in crops, such as corn, sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum officinarum*), is due to plant detoxification mechanisms. Triazine herbicides can be metabolized by plants in three different ways: non-enzymatic hydrolysis of the 2-chloro group, *N*-dealkylation, and GSH conjugation (Shimabukuro et al. 1978). The primary route of metabolism of triazine herbicides in tolerant crops is conjugation with GSH (Lamoureux et al. 1972). The GST that catalyzes this reactions is also present in tolerant sorghum, corn, and sugarcane, but is absent in susceptible species such as peas (*Pisum sativum*), oats (*Avena sativa*), barley (*Hordeum vulgare* L.) and redroot pigweed (*Amaranthus retroflexus*) (Frear and Swanson 1970).

1.4.3 Mode of Action and Herbicidal Characteristics of Glyphosate

Glyphosate (N-(phosphonomethyl)glycine) is a non-selective, foliar-applied herbicide with systemic properties. At the plant surface, glyphosate is absorbed quickly (Caseley and Coupland 1985; Kirkwood et al. 2000) and upon entering the sieve-tube element, moves through the phloem from source to sink, arriving at meristematic tissues in both roots and shoots (Sprankle et al. 1973; Dewey 1982). Glyphosate's target site is present only in some bacteria, fungi, and plants, resulting in very low mammalian toxicity. Due to the moderately short half-life and little movement through the soil, glyphosate is one of the more environmentally benign herbicides (Duke and Powles 2008). Glyphosate inhibits the enzyme EPSPS, which catalyzes the formation of 5-enolpyruvyshikimate-3-phosphate (EPSP) from

shikmate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) in the shikimic acid pathway (Steinrücken and Amrhein 1980).

1.5 Research Objectives

Resistance to herbicides in weeds is an increasing problem that threatens global food security (Délye et al. 2013). Understanding the means by which weeds inherit herbicide resistance traits is critical to gain beneficial insights into resistance evolution, understanding adaptation dynamics and eventually developing superior management practices. Previous research has demonstrated that NTSR mechanisms commonly endow glyphosate resistance. There are only a few reports of glyphosate resistance being conferred by TSR in goosegrass. One of these reports was for a population of goosegrass from Malaysia (Baerson et al. 2002). The Malaysian population also reportedly contained at least one more glyphosate-resistance mechanism, in addition to TS modification. Therefore, when a population of goosegrass from Tennessee was characterized as glyphosate resistant based on whole-plant greenhouse studies and shikimate accumulation (Mueller et al. 2011), research (detailed in Chapter 2) was needed to determine if resistance was TS- or NTS-based and if more than one mechanism was responsible for the resistance.

In the summer of 2009, a waterhemp population (MCR, McLean County resistant) was not sufficiently controlled following foliar applications of HPPD and PSII inhibitors (Hausman et al. 2011). Research later indicated that resistance to both mesotrione and atrazine was via NTSR by elevated rates of herbicide metabolism (Ma et al. 2013). Therefore, in Chapter 3

research with the MCR population by investigating the NTSR inheritance patterns and genetics of mesotrione and atrazine resistances.

1.6 Attributions

Dr. Lawrence Steckel provided the Tennessee parental goosegrass populations (TennGR and TennGS) discussed in Chapter 2. Confirmation of glyphosate resistance in TennGR was confirmed previously by Mueller et al. (2011). The primers used in the PASA assay for determining the genotypes of the TennGR, TennGS, F_1 and F_2 plants were designed by Kaundun et al. (2008). Primers used in sequencing the entire mature protein-coding region of the EPSPS gene were designed with the help of Dr. Chance Riggins and Ahmed Sadeque. Dr. Adam Davis provided statistical analysis. The material presented in Chapter 2 will be submitted for publication with Chance Riggins, Lawrence Steckel and Patrick Tranel as coauthors.

All the seed lines (R, F_1, F_2 and BC_S) for the genetic and inheritance studies in Chapter 3 were prepared by Nick Hausman. Technical assistance with greenhouse experiments was provided by Doug Maxwell and Lisa Gonzini. Dr. Adam Davis provided statistical analysis and advice using R software. Drs. Frederic Kolb and Brian Diers provided advice on genetic inheritance. Chapter 3 will be submitted for publication with Nicholas Hausman, Aaron Hager, Dean Riechers and Patrick Tranel as coauthors.

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CHAPTER 2

The EPSPS Pro106Ser Substitution Solely Accounts for Glyphosate Resistance in a Goosegrass Population from Tennessee

2.1 Abstract

Goosegrass [*Eleusine indica* (L.) Gaertn.] is a problematic summer annual weed that has a strong tendency to evolve resistance to herbicides. Previous studies have documented the occurrence of glyphosate-resistant goosegrass and, in at least some cases, resistance is due to an altered target site. Research was performed to determine if an altered target site was responsible for glyphosate resistance in a Tennessee goosegrass population (TennGR). DNA sequencing of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene revealed a mutation in TennGR plants conferring the Pro106Ser substitution previously identified in other glyphosate-resistant populations. F_1 plants were obtained by crossing TennGR plants with plants from a glyphosate-susceptible population (TennGS). The F_1 hybrids were selfed and two separate F_2 populations were analyzed for their response to glyphosate and also genotyped at the EPSPS locus by PCR amplification of specific alleles (PASA). Plants from the F_2 populations segregated 1:2:1 sensitive:intermediate:resistant in response to a selective dose of glyphosate, and these responses co-segregated with the EPSPS genotypes (PP106, PS106, and SS106). To separately investigate the effect of the Pro106Ser substitution on glyphosate resistance, dose response curves and ED_{50} values were obtained and compared among the three genotypes and the two parental populations. The SS106 genotype was 3.4-fold resistant to glyphosate relative to the PP106 genotype, identical to the resistance level observed for the resistant parental population relative to the sensitive parental population. Based on the results of this study, we

conclude that the mutation conferring a Pro106Ser EPSPS mutation is solely responsible for glyphosate resistance in the TennGR goosegrass population.

2.2 Introduction

Glyphosate inhibits the enzyme EPSPS, which catalyzes the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) from shikimate-3-phosphate (S3P) and phosphoenolpyruvate in plants and select microorganisms, (PEP) (Steinrücken and Amrhein 1980). Various biochemical responses occur when EPSPS is inhibited: (1) decrease of energy in the form of adenosine 5'-triphosphate (ATP), (2) depletion of the essential biomolecules synthesized from the shikimic acid pathway, and (3) substantial carbon flux away from other important pathways by accumulation of shikimic acid (Kaundun et al. 2008).

Glyphosate's success as an herbicide is attributed to many advantageous characteristics, such as: efficient uptake/translocation, low mammalian toxicity, inexpensive production, and broad spectrum activity (Caseley and Coupland 1985; Duke and Powles 2008). Before 1996, resistance to glyphosate was assumed to evolve at lower frequencies when compared to other herbicide families due to glyphosate's unique mode of action and limited metabolism in plants (Bradshaw et al. 1997). Since the introduction of transgenic crops resistant to glyphosate, an increased reliance upon glyphosate for weed control has led to an accelerated evolution of weed populations that are resistant to this herbicide. To date, 32 weeds species in 25 countries have resistance to glyphosate (Heap 2015).

Currently, the identified mechanisms of glyphosate-resistance include both target-site and non-target-site changes. Glyphosate resistance due to a point mutation conferring a

Pro106Ser substitution in EPSPS was first documented in a Malaysian goosegrass [*Eleusine indica* (L.) Gaertn.] population (Baerson et al. 2002) and later in other weed species (e.g., Perez-Jones et al. 2007; Jasieniuk et al. 2008; Bell et al. 2013; Nandula et al. 2013). Other resistance-conferring substitutions in EPSPS have been documented, including Pro106Thr, Pro106Ala, and Pro106Leu (Ng et al. 2003; Wakelin and Preston 2006; Yu et al. 2007; Kaundun et al. 2011; González-Torralva et al. 2014). An alternative target-site-based mechanism of glyphosate resistance, amplification of the EPSPS gene, was first documented in Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Gaines et al. 2010). This mechanism was subsequently documented in other weed species (e.g., Tranel et al. 2010; Salas et al. 2012; Lorentz et al. 2014; Nandula et al. 2014; Wiersma et al. 2015). Non-target-site-based resistances in glyphosate include reduced herbicide uptake and translocation (including herbicide sequestration). A population of ryegrass (*Lolium rigidum* Gaudin) from Australia demonstrated the first case of altered translocation, where glyphosate was found to accumulate in the leaf tips of the resistant plants (Lorraine-Colwill et al. 2002). More recently, vacuolar sequestration of glyphosate was demonstrated in various species of ryegrass (*Lolium* spp.) (Ge et al. 2012). Restricted glyphosate uptake also has been proposed as the mechanism of glyphosate resistance in populations of Palmer amaranth, waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea and Tardif], and johnsongrass [*Sorghum halepense* (L.) Pers.]) (Vila-Aiub et al. 2012; Sammons and Gaines 2014). In general, glyphosate-resistance mechanisms confer relatively low levels of resistance (Sammons and Gaines 2014). Likely as a consequence of this, multiple glyphosate-resistance mechanisms are sometimes found within

individual weed populations (Yu et al. 2007; Dinelli et al. 2008; Kaundun et al. 2011; Nandula et al. 2013; Chatham 2014).

Goosegrass is an annual self-pollinating grass species commonly found in Asia, Africa, South America, and in parts of North America (Holm et al. 1977). Goosegrass is listed as one of the five most troublesome weeds in the world and has high reproductive capabilities, with a single plant capable of producing over 40,000 seeds (Holm et al. 1977). Goosegrass has a history of evolving resistance to numerous herbicides (Heap 2015), including acetyl-CoA carboxylase inhibitors (Leach et al. 1995), bipyridiliums (Buker et al. 2002), glyphosate (Lee and Ngim 2000), glutamine synthase inhibitors (Jalaludin et al. 2010) and dinitroaniline herbicides (Mudge et al. 1984).

A population of goosegrass from Tennessee was characterized as glyphosate resistant based on whole-plant greenhouse studies and shikimate accumulation (Mueller et al. 2011). One of the objectives of this study was to determine if the Tennessee glyphosate-resistant population had a target-site mutation that previously had been associated with glyphosate resistance in other goosegrass populations. If such a mutation was found, a second objective was to determine if it solely accounted for resistance in the population.

2.3 Materials and Methods

2.3.1 Plant Culture

The originating populations used in this study were the glyphosate-resistant and glyphosate-sensitive populations described by Mueller et al. (2011) and herein referred to as TennGR and TennGS, respectively. TennGR was confirmed to be resistant through a whole-plant

dose response and a shikimate assay (Mueller et al. 2011). Progeny (F_1 and F_2 populations) were derived from TennGS and TennGR (see section 2.6) and also used in this study.

Seeds were germinated in 12 x 12-cm trays and seedlings transplanted into 720 cm^3 pots when they had 3–4 true leaves. The growth medium consisted of 3:1:1:1 mixture of LC1 (Sunshine Mix #1/LC1, Sun Gro Horticulture, 770 Silver Street, Agawam, MA 01001), soil, peat and torpedo sand. Slow-release complete fertilizer (Osmocote 13-13-13 slow release fertilizer, The Scotts Company, 14111 Scottslawn Rd., Marysville, OH 43041) was mixed with the growth medium prior to planting, and additional fertilizer was added to the top of the growth medium as needed. Greenhouse conditions were maintained at 28/22°C day/night with a 16:8h photoperiod. Natural sunlight was supplemented with mercury halide lamps to provide a minimum of 800 µmol m^{-2} s^{-1} photon flux at plant canopy level in the greenhouse.

2.3.2 Evaluation of Herbicide Response

Herbicide applications were made using a compressed air research sprayer (DeVries Manufacturing, 86956 State Highway 251 Hollandale, MN 56045) fitted with a Teejet 80015 EVS nozzle (Teejet Technologies, P.O. Box 7900 Wheaton, IL 60187) calibrated to deliver 185 L ha^{-1} at 275 kPa. The nozzle was maintained at 45 cm above the plant canopy. Plants were treated when they were 8-cm tall (measuring from plant base to tallest leaf blade, taking an average of various tillers). Roundup WeatherMax was the formulation used in all experiments (Monsanto, 800 N. Lindbergh Blvd. St. Louis, MO 63167).

2.3.3 DNA Extraction

Harvested leaf material (e.g., TennGS, TennGR, F_1, and F_2 plants) for DNA extraction was either screened with PCR amplification of specific alleles (PASA) for genotyping or used in sequencing reactions. DNA was extracted based on the CTAB method described by Doyle and Doyle (1990). The quality and quantity of the genomic DNA was examined using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, 81 Wyman St., Waltham, MA 02451).

2.3.4 EPSPS Gene Sequencing

The entire mature protein-coding region of the EPSPS gene of four TennGR plants and four TennGS plants was sequenced. TennGR plants were confirmed resistant with a dose of glyphosate lethal to TennGS plants. A fragment containing codon 106 was amplified using primers described previously (Kaundun et al. 2008), using the forward primer EPSPS-SeqF1 (CTCTTCTTGGGGAATGCTGGA) and the reverse primer EPSPS-SeqR1 (TAACCTTGCCACCAGGTAGCCCTC). Other fragments were amplified using primers in Table 2.1, which were designed based on a goosegrass EPSPS gene sequence (GenBank accession AY157642). PCR was conducted in 25 μL reactions with 5 μL of 5X GoTaq green buffer (Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711), 0.2 μL of GoTaq DNA polymerase at 5 U μL^{-1} (Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711), 2 μL of dNTP at 2.5 mM, 2.5 μL $MgCl_2$ at 25 mM, 1 μL of each primer at 10 μM, 1 μL of template DNA, and 12.3 μL purified water. PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles at 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 2 min; and a final extension cycle of 72

°C for 10 min. An aliquot of each PCR was separated on 1% agarose gel (Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547) containing 1 μL ml^{-1} ethidium bromide and visualized using ultraviolet light to confirm amplification of the correct bands. The remainder of each PCR was purified (E.Z.N.A. Cycle Pure Kit, Omega Bio-Tek., 400 Pinnacle Way, Suite 450, Norcross, GA 30071) and used in a sequencing reaction (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems Inc., 850 Lincoln Centre Drive Foster City, CA 94404). The same primers used for amplification were used for sequencing. Sequencing products were analyzed using an AB 3730xl DNA analyzer (Applied Biosystems Inc.). Sequence data were compared and aligned with goosegrass EPSPS gene sequences from glyphosate-sensitive and glyphosate-resistant populations available in GenBank (AY157642 and AY157643) with MEGA6 (Tamura et al. 2013).

2.3.5 PASA Method for Genotyping in TennGR, TennGS, F_1 and F_2 Plants

Four primers were described previously (Kaundun et al. 2008) for PCR amplification of specific alleles (PASA) analysis of the goosegrass EPSPS gene. These primers included two external non-allele specific primers, PASA-F1 (ACAAAGCTGCCAAAAGAGCGGTAG) and PASA–R1 (TAACCTTGCCACCAGGTAGCCCTC), and two allele specific primers, PASA-P (GAATGCTGGAACTGCAATGCGTC) and PASA-S (GCAGCAGTTACGGCTGCTGTCAATTA). With all four primers in one reaction, identifying the wild-type homozygous genotype (PP106), the mutant heterozygous genotype (PS106) and the mutant homozygous genotype (SS106) was possible based on presence/absence of 320-bp and 136-bp fragments. PCR was conducted with the same constituents as previously mentioned, but with 11.3 μL of water and quantity and

concentration of the primers as follows: 0.5 μL each of PASA-F1 and PASA-R1, each at 5 μM; and 1 μL each of PASA-P and PASA-S, each at 10 μM. PCR conditions were as follows: initial denaturation at 95 °C for 2 min; 24 cycles with 95 °C for 30 sec, 65 °C for 30 sec (-1 °C per cycle), and 72 °C for 45 sec; and a final extension cycle of 72 °C for 4 min. Amplicons were separated on a 2% agarose gel containing 0.5 μl ml^{-1} ethidium bromide and visualized with UV light.

2.3.6 Generation of F_1 and F_2 Plants

Prior to crossing to make F_1s, leaf samples for DNA extraction of TennGR plants were collected for genotyping using the PASA protocol (described above) to ensure plants were homozygous resistant (SS106). Since goosegrass is primarily self-pollinating, crossing to make F_1 plants was done by emasculating selected florets (Richardson 1958) before anthesis of the TennGS maternal parent flowers with precision tweezers (Excelta 3C-SA-ET, Excelta Corporation, 60 Easy Street, Buellton, CA 93427). All TennGS maternal parent flowers not emasculated were discarded from the plant. After emasculation, florets from the TennGS were tied together with selected florets from a TennGR paternal plant and enclosed within a glassine bag to prevent contamination by unwanted pollen. Twenty-three seeds were harvested from the crosses and eighteen of these were planted in the greenhouse. Tissue samples from newly emerging leaves were screened with the PASA assay to ensure plants were heterozygous (PS106). Of the eighteen plants, 100% were heterozygous. Nine plants were grown in isolation and allowed to self-pollinate to produce F_2 populations. Two F_2 populations were arbitrarily selected for further characterization.

2.3.7 Segregation of F_2

Greenhouse studies were performed to determine if the resistance trait co-segregated with the Pro106Ser mutation. Preliminary studies concluded that a glyphosate rate of 350 g ae ha^{-1} best discriminated between parental populations. Two populations (termed D1 and B1) of F_2 seedlings (89 plants for D1 and 96 for B1) of uniform size (8–10 cm) were evaluated. At 14 days after treatment (DAT), individual plants were visually evaluated and rated as sensitive (complete mortality/very limited green tissue), intermediate (green tissue but reduced tillering compared to controls), or resistant (no reduced tillering). A chi-square goodness of fit test (χ^2) was used to compare the observed and expected plant resistance frequencies based on a single gene model. The single gene model was rejected if $p<0.05$. Tissue samples for DNA extraction were taken from plants prior to herbicide application for genotyping using the PASA protocol. Genotypes and glyphosate response phenotypes were compared for each individual F_2 plant.

2.3.8 F_2 Dose Response Comparison of the Three Segregating EPSPS Genotypes

A glyphosate whole-plant dose response was performed on individual genotypes to compare the parental populations with the segregating F_2 populations. Prior to herbicide application, tissue from a newly emerging leaf was obtained from each F_2 plant for DNA extraction and PASA analysis. As in above, a chi-square goodness of fit test (χ^2) was used to compare the observed and expected plant resistance frequencies based on a single gene model. The single gene model was rejected if $p<0.05$. Uniformly sized plants of 8–10 cm were selected from F_2, TennGR, and TennGS populations and treated with various rates of glyphosate (0, 105, 210, 420, 840, 1,680, 3,360 and 6,720 g ha^{-1}). After herbicide application, plants were

returned to the greenhouse and placed in a completely randomized design. Six plants of each parental population and at least 16 plants of each of the two F_2 populations were treated with each glyphosate dose. At 21 DAT, plant injury was visually evaluated and recorded using a scale ranging from 0 (no green tissue) to 100 (no injury). Aboveground plant tissue was harvested and dried at 65 °C for at least five days before dry weights were recorded. The dry weight data (m) and the visual data (v) were combined to obtain an adjusted dry weight (y) using the function:

$$y = m \text{ x } v/100$$

Adjusted dry weights were expressed relative to the mean of the corresponding population's no-herbicide control.

Based on PASA analysis, each F_2 population was subdivided into three genotypes PP106, PS106, and SS106. The data were analyzed using a non-linear regression model with the dose-response curve package in R software (Knezevic et al. 2007) based on the following equation:

$$y = c + \frac{d-c}{1+\exp\{b[\log(x)-\log(ED_{50})]\}}$$

This is a four-parameter non-linear logistic dose response model where *b* is the slope of the curve, *c* is the lower limit, *d* is the upper limit and the ED_{50} value is the herbicide dose causing a 50% reduction in adjusted dry weight. Resistance ratios were calculated as the ED_{50} of the TennGR, SS106, or PS106 population/genotype divided by the ED_{50} of the TennGS or PP106 population/genotype.

2.4 Results

2.4.1 EPSPS Gene Sequence

Sequences that spanned the entirety of the EPSPS gene coding region, as well as the non-coding regions, were obtained from four TennGR plants that survived glyphosate at a dose that was lethal to TennGS plants, and were compared to TennGS sequences. Within each of the TennGR and TennGS groups, the four sequences obtained were identical. A total of three polymorphisms were observed in the TennGR coding region relative to the TennGS sequence (Figure 2.1). Two of the three polymorphisms in the coding region did not change the corresponding amino acid residue. The third polymorphism conferred a Pro106Ser amino acid substitution, which corresponds to a previously documented glyphosate-insensitive goosegrass EPSPS (GenBank AJ417033) (Baerson et al. 2002). Within the non-coding regions, five polymorphisms (all of which were single nucleotide substitutions) were observed between the TennGR and TennGS sequence (data not shown).

2.4.2 Segregating of F_2

Segregation of glyphosate resistance was evaluated at a glyphosate dose which best discriminated the parental populations. Segregation in both F_2 populations (B1 and D1) evaluated did not deviate from the 1:2:1 (R:I:S) ratio expected for a single gene ($p=0.6$, $p=0.1$ for D1 and B1 respectfully). Furthermore, the glyphosate response and EPSPS genotype were associated: 70–80% of the plants with the mutant homozygous genotype were visually rated as resistant, 82–93% of the plants with the heterozygous genotype were visually rated as

intermediate, and 100% of the plants with the wild-type homozygous genotype were visually rated as sensitive (Figure 2.2).

2.4.3 F_2 Dose Response Comparison of the Three Segregating EPSPS Genotypes

A whole plant dose response analysis of each genotype present in the F_2 populations indicated that there was lack of significant difference in the ED_{50} values of the parental TennGS population (95 g ha^{-1}) and the PP106 genotype (119 g ha^{-1}), or in the ED_{50} values of the parental TennGR population (320 g ha^{-1}) and the SS106 genotype (399 g ha^{-1}) (Figure 2.3; Table 2.2). The resistance ratio of the TennGR parent relative to the TennGS parent was 3.4. The SS106 genotype also had a resistant ratio of 3.4 relative to the PP106 genotype. Overall, the response of the PS106 genotype was intermediate between those of the PP106 and SS106 genotypes, giving a resistant ratio of 2.3 relative to the PP106 genotype. Degree of dominance (D) was calculated for PS106 genotype using the formula given by Stone (1968) and results indicated that glyphosate resistance is nearly additive (D=-0.01). Segregation of the EPSPS gene in both F_2 populations (B1 and D1) evaluated did not deviate from the expected 1:2:1 ratio (p=0.7, p=0.2 for D1 and B1 respectfully).

2.5 Discussion

2.5.1 EPSPS Gene Sequence

Sequence comparison for the TennGR population to the TennGS population showed a total of 8 polymorphisms, but five of the eight mutations appeared within introns. The differences seen in the EPSPS gene introns were mostly at a position far from the intron/exon

borders and were not within the splice junction regions (Ng et al. 2004). Consequently, we expect that the mutations do not affect splicing and, therefore, do not affect enzyme activity. Of the three polymorphisms seen in the exons, two occurred at the third nucleotide position of a codon and did not change the amino acid residue. The only nonsynonymous polymorphism found in the TennGR population conferred a Pro106Ser substitution, which has been documented previously to confer glyphosate resistance in many weed species (Baerson et al. 2002; Perez-Jones et al. 2007; Jasieniuk et al. 2008; Bell et al. 2013; Molin et al. 2013; Nandula et al. 2013).

Few locations in the EPSPS enzyme are likely to confer target-site resistance without a significant fitness penalty because of the select and highly conserved amino acids that bind to glyphosate (Mizyed et al. 2003). However, the EPSPS codon at position 106 is now well documented to contain polymorphisms that confer low levels of glyphosate resistance, around 2- to 4-fold resistance (Baerson et al. 2002; Wakelin and Preston 2006; Jasieniuk et al. 2008). Changing an amino acid at codon 106 causes the structural configuration of the active site to change, forcing other amino acids to move towards the inhibitor, reducing the available space in the active site (Sammons and Gaines 2014). Enzyme kinetics performed with a Pro106Ser mutation showed a decrease in the affinity for PEP, but subsequent research on goosegrass EPSPS indicated that the loss in substrate binding may not be as severe as initially suspected (Baerson et al. 2002).

2.5.2 Segregation of F_2

Chi-square analysis of the segregating F_2 populations supported a single gene model for glyphosate resistance. Furthermore, phenotypic segregation was strongly associated with the segregation of Pro/Ser at codon 106 of EPSPS. All plants phenotyped as resistant contained at least one Pro106Ser allele, and 70–80% were homozygous for this allele. None of the plants genotyped as homozygous for the wild type EPSPS allele were phenotyped as intermediate or resistant, indicating that there was not another resistance mechanism in the population. To further rule out the presence of another resistance mechanism in the TennGR goosegrass population, a separate experiment was performed to generate dose-response curves for each of the three EPSPS genotypes within the F_2 populations.

2.5.3 F_2 Dose Response Comparison of the Three Segregating EPSPS Genotypes

The TennGR population was 3.4-fold resistant to glyphosate when compared to the TennGS population. The magnitude of glyphosate resistance observed herein for the TennGR population is similar to that reported for other glyphosate-resistant populations endowed by the same substitution (Baerson et al. 2002; Ng et al. 2004; Jasieniuk et al. 2008). Mueller et al. (2011) previously reported that glyphosate resistance in the TennGR population was 7.4-fold relative to TennGS. Although this is about twice that observed herein, our study used adjusted dry weights (factoring in visual observations) whereas their resistance magnitude was based solely on dry weights. Kaundun et al. (2008) reported that the Pro106Ser EPSPS mutation conferred about a two-fold level of glyphosate resistance when present in the homozygous state in goosegrass. According to the field history of the Tennessee population (Mueller et al.

2011), the use of below-label glyphosate dosages occurred for several years. Others have also reported that reduced-rate herbicide applications can accelerate the selection of resistant populations (e.g., Baerson et al. 2002; Manalil et al. 2011; Busi et al. 2012).

The two homozygous genotypes in the F_2 population, PP106 and SS106, exhibited dose response curves that were similar to those of their corresponding parental populations, TennGS and TennGR, respectively. Consequently, the resistance ratio of PP106 relative to SS106 was identical to that of TennGR relative to TennGS. If another factor contributing to resistance was present in the TennGR population some plants genotyped as PP106 should contain this factor (absent strong genetic linkage), decreasing glyphosate sensitivity relative to the parental TennGS population. By the same logic, some plants genotyped as SS106 should lack the second factor, increasing their glyphosate sensitivity relative to the parental TennGR population.

Kaundun et al. (2008) similarly used PASA to compare glyphosate resistance among homozygous sensitive and resistant genotypes. Although they used a segregating field population from the Philippines, rather than an experimentally derived F_2 population as done herein, they also concluded that the target-site mutation was the major factor conferring resistance. Unlike in our study, however, they observed a small but statistically significant decrease in glyphosate sensitivity of the PP106 genotype relative to their sensitive control population, suggesting the presence of one or more minor-effect genes.

EPSPS target-site mutations have been documented in six weed species, and are most frequently reported in the genus *Lolium* (Gaines and Heap 2015). Since *Lolium* spp. are commonly out-crossing species, the likelihood of accumulating multiple glyphosate-resistant genes/alleles is very probable. For example, rigid ryegrass and perennial ryegrass (*Lolium*

perenne L.) populations have been separately documented to have a target site mutation in EPSPS and reduced glyphosate translocation (Bostamam et al. 2012; Ghanizadeh et al. 2015). In studies involving glyphosate-resistant goosegrass, a second mechanism of resistance was suspected (Ng et al. 2004; Kaundun et al. 2008), but more research is needed to confirm this. The self-pollinated nature of goosegrass reduces the likelihood that this species will accumulate multiple resistance mechanisms.

Glyphosate-resistant goosegrass has now been documented in multiple countries and in multiple U.S. states (Heap 2015). Although the Pro106Ser EPSPS substitution confers a very modest level of glyphosate resistance in goosegrass, it is significant enough to confer field-level resistance. Continued reliance on glyphosate likely will lead to additional glyphosate-resistant goosegrass populations, and possibly to the selection of other major glyphosate-resistance mechanisms in this species. In fact, a double mutation (Pro106Ser and Thr102Ile) in goosegrass EPSPS, which confers strong resistance to glyphosate, was recently reported (Yu et al. 2015).

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2.7 Tables and Figures

Table 2.1: Primers used for EPSPS gene amplification and sequencing.

Primer name	Sequence (5’ → 3’)
E.coracana_Forward	GCTCGGCTGTGGTGGT
Rev-epsps-x1/2-f2	GGACAAAGCTGCCAAAAGAG
epsps-utr5-F1	CTCGCCGAGGTAAGAAGAAG
epsps-x1/1-R1	GTAGTTGTTGGCTGTGGTGG
epsps-x1/2-F2	GGACAAAGCTGCCAAAAGAG
epsps-x2-R2	CTTTAGCTCTTGGGGATGTGG
epsps-in2-F3	TCCTTTTGGGCTGGTGTTAG
epsps-x3-R3	TGGAGGGACTGTGACTGTTG
epsps-in3-F4	GCCAGTCATTTTGTTCTCAGC
epsps-x4-R4	GATGATGGGAGCGAAGGTTA
epsps-x4-F5-2	GGAGCGAAGGTTACATGGACT
epsps-x6-R5-2	CACCTACGATGACCACAGGAT
epsps-x4-F6	ATGAACAAAATGCCCGATGTC
Goose_FinalREV	CTAAACTGCGTCTGTGCCTG

Table 2.2: Whole-plant responses to glyphosate of parental TennGS and TennGR populations, and three genotypes segregating within two combined F_2 populations.

Population	ED_{50}[1] ($g\ ha^{-1}$)	R/S[2]
Parental Lines		
TennGS	95 (17.9)[3]	1
TennGR	320 (58.8)	3.4
Segregating F_2		
PP106	119 (10.9)	1
PS106	216 (16.4)	2.3
SS106	399 (80.1)	3.4

[1] The effective dose at which plants show a 50% reduction, which was determined using a combination of dry weights and visual observations of herbicide responses.

[2] ED_{50} of parental TennGR, SS106 genotype or PS106 genotype divided by ED_{50} of the corresponding sensitive population.

[3] Numbers in parentheses denote ±1 s.e.

Figure 2.1: Sequence comparison of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene from glyphosate-resistant (TennGR) and sensitive (TennGS) plants. Three polymorphisms were found in the exons. Regions of homology are indicated by dots. Numbering is consistent with that used previously for goosegrass EPSPS (GenBank AJ417033) from Baerson et al. 2002.

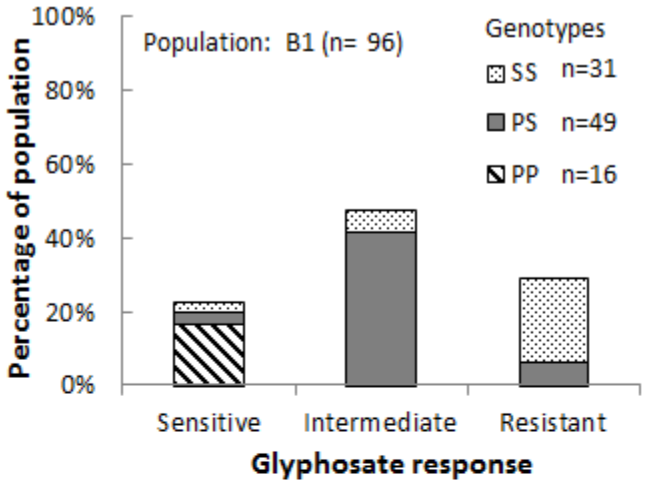
```
          103   A    M    R   S/P   L    T   108
TennGR:       GCA  ATG  CGA  TCA  TTG  ACA
TennGS:       ...  ...  ...  C..  ...  ...

          142   Q    L    G    A    D    V   147
TennGR:       CAG  CTT  GGT  GCG  GAT  GTT
TennGS:       ...  ...  ...  ..A  ...  ...

          197   E    I    E    I    I    D   202
TennGR:       GAG  ATT  GAA  ATC  ATT  GAT
TennGS:       ...  ...  ...  ..A  ...  ...
```

Figure 2.2: Co-segregation of the resistance trait with Pro106Ser mutation. The responses of plants from two different F_2 populations, (a) B1 (n= 96) and (b) D1 (n= 89) were visually evaluated at a single rate of glyphosate (350 g ha^{-1}). Tissue samples for DNA extraction were taken from plants prior to herbicide application for genotyping using the PCR amplification of specific alleles (PASA) protocol. Genotypes are represented by a different pattern within the columns.

(a)



(b)

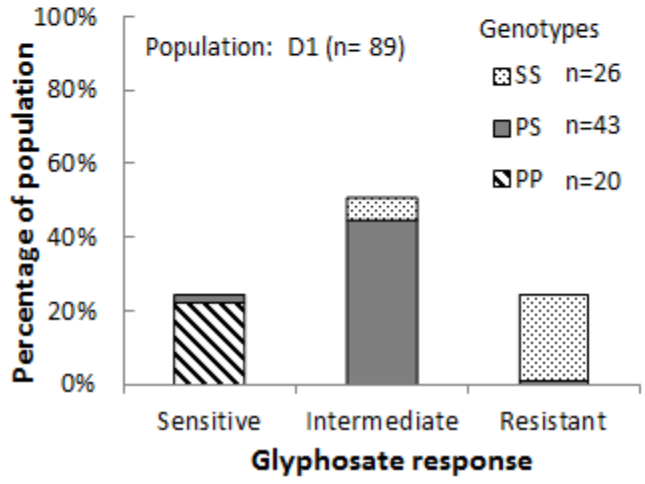
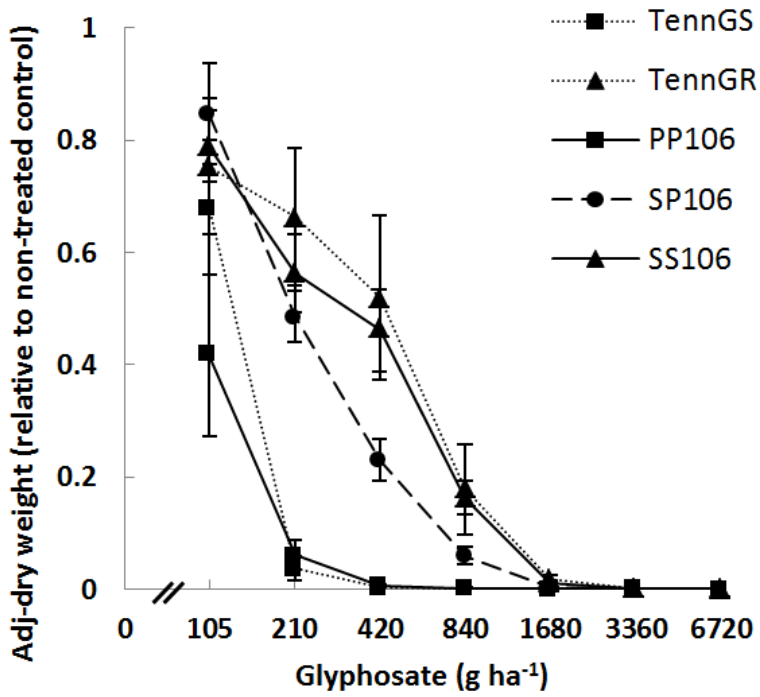


Figure 2.3: Glyphosate dose-response curves for parental TennGS and TennGR populations, and three genotypes segregating within two combined F_2 populations. Vertical bars indicate ±1 s.e.



CHAPTER 3

Genetics and Inheritance of Non-target-site Resistances to Atrazine and Mesotrione in an Illinois Waterhemp Population

3.1 Abstract

A population (designated MCR) of waterhemp (*Amaranthus tuberculatus*) from McLean County, Illinois is resistant to both mesotrione and atrazine by elevated rates of herbicide metabolism. Resistant and sensitive plants were crossed to obtain reciprocal F_1 lines, which were then used to create pseudo F_2 and backcross (to sensitive parent; BC_s) lines. The various lines were evaluated with whole-plant herbicide efficacy studies in a greenhouse. The responses of the F_1 lines to both mesotrione and atrazine were intermediate when compared to parental populations. In the case of atrazine, BC_s and F_2 lines segregated 1:1 and 1:3, respectively, for susceptibility (S): resistance (R), at a dose that controlled the sensitive parent but not the F_1 or resistant parent. For mesotrione, segregation was observed within the F_1 lines, suggesting that mesotrione resistance is multigenic and the resistant parents used in the cross were not homozygous at the resistance loci. Furthermore, at low mesotrione doses, more F_2 plants survived than expected based on a single gene trait, whereas at high doses, fewer F_2 plants survived than expected. Dry weight data confirmed the conclusions obtained from survival data. Specifically, atrazine responses segregated into two discreet classes (R and S) in both the F_2 and BC_S lines, whereas mesotrione responses showed a continuous distribution of phenotypes in F_2 and BC_S lines. We conclude that metabolism-based atrazine resistance in MCR

is conferred by a single major gene, whereas inheritance of mesotrione resistance in this population is complex and likely mediated by two or more unlinked genes.

3.2 Introduction

Waterhemp has been a prevalent agronomic weed species in the Midwest U. S. since the 1990s. Indigenous to Illinois, waterhemp is a dioecious plant with wind pollinated flowers and rapid growth rate due to being C_4 (Steckel 2007). It is a prolific reproducer, with a single female plant capable of producing one million seeds (Steckel et al. 2003). The biological attributes of waterhemp combine to make this species particularly adept at evolving resistance to herbicides (Tranel et al. 2011).

A population (designated MCR, for McLean County Resistant) of waterhemp evolved resistance to atrazine and 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor herbicides (Hausman et al. 2011). Atrazine disrupts electron transport by outcompeting plastoquinone for the secondary electron-accepting plastoquinone-binding site on the D1 protein of photosystem II (PSII) in chloroplasts causing cellular damage by oxidative stress (Hess 2000). Resistance to atrazine and similar PSII inhibitors is common, having been documented in at least 72 weed species (Heap 2014). HPPD-inhibiting herbicides cause the photooxidative damage of chlorophyll and the destruction of photosynthetic membranes in developing shoot tissue, which results in what is commonly referred to as bleaching of new leaf tissue (Mitchell et al. 2001). Resistance to HPPD inhibitors has been reported in only two weed species (Hausman et al. 2011 and Jhala et al. 2014).

There are two general mechanisms of herbicide resistance in plants: (1) target site alterations, such as mutations affecting herbicide binding kinetics or amplification of the target site gene (Powles and Yu 2010), and (2) non-target-site (NTS) mechanisms based on differences in e.g., herbicide metabolism or translocation (including herbicide sequestration) (Yuan et al. 2007; Powles and Yu 2010). Whereas target-site resistance is often conferred by a single gene, non-target-site mechanisms are often multigenic, resulting in complex patterns of inheritance (Délye et al. 2011). Although the appearance of herbicide-resistant weed biotypes has been well documented, information on the inheritance of resistance, particularly for NTS resistance, is largely unavailable (Lorraine-Colwill et al. 2001). Inheritance of herbicide resistance provides insights into resistance evolution, genetic structure of weed populations, adaptation dynamics, and resistance management (Neve et al. 2009).

Previous research on the MCR population indicated that resistances to both atrazine and mesotrione are mediated by NTS mechanisms. Specifically, enhanced herbicide metabolism by cytochrome P450 and glutathione *S*-transferase (GST) activity conferred resistance to mesotrione and atrazine, respectively (Ma et al. 2013). The objective of this research was to determine the inheritance of these two resistance traits and gain insight into the number of genes involved.

3.3 Materials and Methods

3.3.1 Parental, F_1, Pseudo F_2 (ψ-F_2) and Backcross (BC) Plants

The originating populations used in this study were MCR and WCS respectively. The former was described by (Hausman et al. 2011) and is resistant to both mesotrione and

atrazine, and the latter is known to be sensitive to these herbicides (Patzoldt et al. 2006). Plants from the original MCR field collection were grown in a greenhouse and selected with an application of an HPPD inhibitor [105 g mesotrione ai ha^{-1} (Callisto, Syngenta P.O. Box 18300 Greensboro, North Carolina 27419) or 18 g topramezone ae ha^{-1} (Impact, AMVAC 4100 E. Washington Blvd. Los Angeles, California 90023)] plus atrazine [560 g ai ha^{-1} (AAtrex 4L, Syngenta)] when they were 10–15 cm tall. Survivors were used as resistant (R) parents for crosses. Two pairwise crosses, each between two R parents, yielded progenies designated NH2 and NH3, which were used as R control populations for dose response and segregation experiments. WCS plants were obtained from previous seed increases of the original WCS field collection, and were used as sensitive (S) parents in crosses and as the S control population in dose response and segregation experiments.

F_1 plants derived from four parental crosses, two R X S and two S X R (female parent listed first), were used in the studies. Plants from F_1 lines that survived mesotrione (158 or 210 g ha^{-1}, applied to 10–15 cm plants) were utilized in subsequent crosses. The dioecious nature of waterhemp precludes selfing of F_1s to make true F_2s; therefore, F_1 plants were intermated to make ψ-F_2 lines (hereafter referred to simply as F_2 lines). F_1 males were allowed to also pollinate WCS females to produce BC_s (backcross to S) lines. All crosses were performed in greenhouse rooms, and intermated plants were enclosed within a tent constructed with a 198 cm x 183 cm pollination bag (Vilutis & CO, 22535 S Center Rd, Frankfort, IL 60423). Individual crosses are listed in the supplemental material. Progeny seeds were suspended in 0.1 g L^{-1} agar solution at 4°C for at least 4 weeks to enhance germination (Bell et al. 2013).

3.3.2 Evaluation of Herbicide Response

Seeds from the various lines were germinated on water-saturated filter paper in petri dishes incubated in a germination chamber (CMP4030 model, Conviron 572 South Fifth Street - Suite 2 Pembina, ND U.S.A 58271) set for 15/35°C day/night with a 12:12h photoperiod. Seedlings were transferred into either cone-tainers (Ray Leach SC10 "Cone-tainer", 31933 Rolland Drive, Tangent, Oregon 97389 USA) for segregation analysis, or 12 x 12-cm trays for herbicide dose response experiments. Those planted in 12 x 12-cm trays were later transplanted into 720 cm^3 pots when seedlings were about 2-cm tall. Both the pots and the cone-tainers contained growth medium consisting of 3:1:1:1 mixture of LC1 (Sunshine Mix #1/LC1, Sun Gro Horticulture, 770 Silver Street, Agawam, MA 01001), soil, peat and torpedo sand. Slow-release complete fertilizer (Osmocote 13-13-13 slow release fertilizer, The Scotts Company, 14111 Scottslawn Rd., Marysville, OH 43041) was mixed into the growth medium prior to planting, and additional fertilizer was added to the top of the growth medium as needed. Greenhouse conditions were maintained at 28/22°C day/night with a 16:8h photoperiod. Natural sunlight was supplemented with mercury halide lamps to provide a minimum of 800 µmol m^{-2} s^{-1} photon flux at plant canopy level in the greenhouse.

Herbicide applications were made using a compressed air research sprayer (DeVries Manufacturing, 86956 State Highway 251 Hollandale, MN 56045) fitted with a Teejet 80015 EVS nozzle (Teejet Technologies, P.O. Box 7900 Wheaton, IL 60187) calibrated to deliver 185 L ha^{-1} at 275 kPa. The nozzle was maintained approximately 45 cm above the plant canopy. Plants grown in cone-tainers were sprayed when they were 5–7 cm tall, and plants grown in pots were sprayed when they were 10–15 cm tall. Mesotrione spray solutions contained methylated seed

oil (MSO, 1% v/v) and liquid ammonium sulfate (AMS, 2.5% v/v), while atrazine was applied with crop oil concentrate (COC, 1% v/v).

3.3.3 F_1 Whole Plant Dose Responses

Uniform plants 10–15 cm tall were selected from F_1, R (NH3), and S (WCS) populations and treated with various rates of either atrazine or mesotrione. The rates for mesotrione and atrazine were equally spaced along a logarithmic scale with a base of 3.16. After herbicide application, plants were returned to the greenhouse and placed in a completely randomized design. For atrazine treatments, two runs were used, and each treatment (single plant) was replicated at least four times in the F_1 (NH5 and NH6) and S (WCS) populations. Seed supply was limited for R (NH3) during atrazine treatments so two replicates were used in Run 1 and four replicates were used for Run 2. For mesotrione, three runs were used, and each treatment was replicated six times. For mesotrione, all runs included S (WCS), F_1, and R (NH3) populations. Run 1 and Run 2 included the F_1 populations NH5, NH6, NH9 and NH10; whereas Run 3 included the F_1 populations NH5 and NH6.

At 12 and 21 days after treatment (DAT) for atrazine and mesotrione, respectively, plant injury was visually evaluated and recorded using a scale ranging from 0 (no green tissue) to 100 (no injury). Aboveground plant tissue was then harvested and dried at 65 °C for at least 4 days before dry weights were recorded. The dry weight data (m) and the visual data (v) were combined to obtain an adjusted dry weight (y) using the following function:

$$y = m \text{ x } v/100$$

Adjusted dry weights were expressed relative to the mean of the corresponding population's no-herbicide control.

For both dose responses, a linear model was used to compare the response of each population across runs using R software (R version 3.1.1, R Core Team 2013). There was no significant interaction between run and population, so the combined data were fit to dose response curves for each herbicide. The combined data were analyzed using a non-linear regression model with the dose response curve package in R software (Knezevic 2007) based on the following equation:

$$y = c + \frac{d-c}{1+\exp\{b[\log(x)-\log(ED_{50})]\}}$$

This is a four-parameter non-linear logistic dose response model where *b* is the slope of the curve, *c* is the lower limit, *d* is the upper limit, and the ED_{50} value is the herbicide dose causing a 50% reduction in adjusted dry weight. Resistance ratios were calculated as the ED_{50} of the R or F_1 population divided by the ED_{50} of the S population.

3.3.4 Segregation Analysis in BC_s and F_2 Populations

Preliminary studies were conducted for both atrazine and mesotrione to determine appropriate herbicide rates for segregation analysis. An atrazine rate of 985 g ha^{-1} was chosen because it effectively distinguished S plants from F_1 and R plants. For mesotrione, because of the lower magnitude of resistance and because of a less uniform response of F_1 plants, it was not possible to identify a single high rate that would consistently distinguish F_1 and R plants, nor a single low rate that would distinguish F_1 and S plants. Therefore, multiple rates (from 75 to 120 g ha^{-1} for high rates, and from 4 to 25 g ha^{-1} for low rates) were included in each run, and

the rates that best distinguished F_1 plants (from R plants at the high rates and from S plants at the low rates) were used for segregation analysis. In the end, mesotrione segregation at a high rate was assessed in two runs that included two F_2 lines each at rates ranging from 95–120 g ha^{-1}; and at a low rate (10 g ha^{-1}) in three runs that each included two F_2 lines and two BC_s lines. For each herbicide rate at each run, included BC_s and F_2 lines were each represented by 20–49 and 72–107 plants, respectively. Each rate at each run also include 10–18 S plants, 10–21 F_1 plants and 10–21 R plants. At 12 and 14 DAT for atrazine and mesotrione, respectively, each F_2 and BC_S plant was visually evaluated and assessed as dead or alive (new growth evident).

A chi-square goodness of fit test (χ^2) was used to compare the observed and expected plant survival frequencies based on a single gene model. The single gene model was rejected if $p < 0.05$. For mesotrione, corrections to the expected survival frequencies were made based on observed survival of the F_1 and parental populations at the same mesotrione rate and in the same run, assuming a single gene model (Busi et al. 2012; Han et al. 2014). For example, the expected survival frequency of an F_2 population was calculated as:

$$\text{Exp } F_2 = 0.25 \times \text{Obs R} + 0.5 \times \text{Obs } F_1 + 0.25 \times \text{Obs S}$$

where Obs is the observed frequency of survival in R, F1, or S populations.

In one run of the experiment, all aboveground plant tissue was harvested and dried at 65 °C for at least four days, and dry weights recorded. The dry weight data were used in frequency distribution analysis to better visualize the segregation of the populations. Dry weights of parental lines did not have a normal distribution, but did demonstrate a normal distribution after natural-log transformation (Kolmogorov-Smirnov, p values = 0.21–0.84); therefore, natural log transformations of dry weights were used on all the lines.

3.4 Results

3.4.1 Whole Plant F_1 Dose Response

F_1 lines did not significantly differ from each other in their responses to either atrazine or mesotrione (Tables 3.1 and 3.2). Overall, the responses of the F_1 lines were intermediate to that of the R and S parental populations for atrazine (Figure 3.1). Resistance in the R parent relative to the S parent was 41- and 16-fold for atrazine and mesotrione, respectively. F_1 lines had resistance ratios of 7–11 for atrazine and 4–8 for mesotrione. Degree of dominance (D) was calculated for pooled F_1 lines using the formula given by Stone (1968). Although inheritance of both atrazine and mesotrione resistances were nearly additive (D=0), atrazine resistance was slightly greater than additive (D=0.12) and mesotrione resistance was somewhat less than additive (D=-0.29).

3.4.2 Inheritance of Atrazine Resistance in Segregating Populations

Segregation of atrazine resistance was evaluated at an atrazine dose in which resistance was functionally dominant (i.e., F_1 plants survived). Segregation in both of the F_2 lines evaluated did not deviate from the 3:1 (R:S) ratio expected for a single dominant gene in either of two experimental runs (Table 3.3). Similarly, two BC_S lines did not deviate from the expected 1:1 ratio in either of two runs. One BC_S line (NH53) significantly deviated from the expected 1:1 ratio in the first run (p=0.03) but not in the second run when more plants were evaluated (p=0.09).

Frequency distributions of dry weight data from individual plants showed discreet phenotypic classes, consistent with atrazine resistance being a qualitative trait (Figure 3.2). Dry

weight distributions between the R parent and F_1 population significantly overlapped, comprising a single phenotypic class. This was not surprising since the atrazine rate was chosen such that resistance was functionally dominant. The F_2 population had two phenotypic classes: about 25% of the plants had dry weights similar to those of the S parent and about 75% of the plants had dry weights similar to those of the R parent and F_1 plants. Plants in the BC_S population also exhibited these two phenotypic classes, with about half of the plants in each class.

3.4.3 Inheritance of Mesotrione Resistance in Segregating Populations

As with atrazine, segregation was evaluated at a mesotrione rate at which resistance was functionally dominant. However, due to segregation of the F_1 line (Figure 3.2) and the relatively low level of resistance, it was not possible to find a single rate that absolutely distinguished S and F_1 plants. For this reason, and because preliminary observations suggested multigenic inheritance, segregation of mesotrione resistance also was evaluated at a high rate, at which resistance was functionally recessive.

3.4.3.1 Low Rate Analysis

Although multiple rates were used for the low rate study, 10 g ha^{-1} was the minimum dose that most effectively controlled the S plants in all runs. At this rate, only one S plant (2%) survived in all three runs, whereas 20% of S plants survived at the next lowest rate of 8 g ha^{-1}. Segregation of BC_s and F_2 plants therefore was evaluated only at the 10 g ha^{-1} rate. At this rate, survival of F_1s ranged from 35–76% across runs, and expected survival percentages of BC_s and

F_2 lines were adjusted based on the lack of 100% F_1 survival. Segregation in each F_2 line evaluated deviated from the corrected 3:1 (R:S) ratio expected for a single dominant gene in all three experimental runs (Table 3.4). Similarly, all BC_s lines deviated from the corrected expected 1:1 ratio in all three runs (Table 3.4). Survival of the BC_s lines ranged from 2–17%, which was less than the corrected expected survival percentages (18–41%). In contrast, survival of F_2 plants was higher than the corrected expected (43–65%), ranging from 75–90%.

Frequency distributions of dry weight data from segregating lines did not display discreet phenotypic classes. For example, dry weights of F_2 plants treated with 10 g mesotrione ha^{-1} exhibited a bell-shaped distribution, which spanned almost the entire range of dry weights collectively spanned by plants from the S and R parents (Figure 3.2).

3.4.3.2 High Rate Analysis

Rates in the range of 95–120 g ha^{-1} best distinguished R and F_1 plants and therefore were used for segregation analysis in the F_2 lines. Segregation in each of the F_2 lines evaluated consistently deviated from the corrected 1:3 (R:S) ratio expected for a single recessive gene (Table 3.4). Survival percentages of F_2 lines ranged from 0–5%, which were much less than the 25% expected for a single gene model, even when taking into account the correction for less than 100% survival of R (e.g., Run 1, 120 g ha^{-1}) or more than 0% survival of the F_1 (e.g., Run 2, 95 g ha^{-1}).

3.5 Discussion

3.5.1 Whole Plant F_1 Dose Response

The MCR population was 16- and 41-fold resistant to mesotrione and atrazine, respectively, when compared to the sensitive population. Even though atrazine resistance in the MCR population was reported previously (Hausman et al. 2011), the magnitude of resistance was not reported. The magnitude of atrazine resistance observed herein for the MCR population is similar to that reported for ACR (38-fold) and SegR (16-fold), two other waterhemp populations with NTS atrazine resistance (Patzoldt et al. 2003; 2005), and much less than that observed (>1000-fold) in waterhemp populations with target-site atrazine resistance (Foes et al. 1998; Patzoldt et al. 2003).

Hausman et al. (2011) previously reported that mesotrione resistance in the MCR population was 35-fold relative to WCS. Although this is about twice that observed herein, our study used adjusted dry weights (factoring in visual observations) whereas their resistance magnitude was based solely on dry weights. The apparent multigenic nature of mesotrione resistance in MCR (observed in the segregation analysis), along with the outcrossing biology of waterhemp, confounds quantifying the resistance magnitude. In fact, depending on the number of resistance loci present in the population, recurrent mesotrione selection of the population may continue to increase the resistance magnitude for multiple generations (Neve and Powles 2005). Segregation of mesotrione resistance in the F_1 lines (even though highly resistant R parent plants were selected for the crosses) is evidence that the R parents are still segregating at one or more resistance loci.

The responses of the F_1 lines were intermediate to that of the R and S parental populations for both atrazine and mesotrione (Figure 3.1). Resistance to mesotrione in reciprocal F_1 lines did not differ, indicating mesotrione resistance is nuclear inherited. In the case of atrazine, the dose response was performed on F_1 lines from only one crossing direction (R parent as female). However, resistance in BC_S lines, obtained from crosses in which the F_1 was the male parent, indicate that atrazine resistance also is nuclear inherited.

Based on the degree of dominance calculations, atrazine resistance can be described as incompletely dominant whereas mesotrione resistance can be described as incompletely recessive (Stone 1968). However, the degree of dominance for mesotrione may not have been accurately revealed in our study, given the above mentioned caveat of multigenic mesotrione resistance coupled with lack of homogeneity of the parental R plants.

From a practical standpoint, both atrazine and mesotrione resistance in MCR functionally can behave as a dominant or recessive trait, depending on the herbicide rate (as well as other factors such as plant size at time of application). The relatively high magnitude of atrazine resistance in the MCR population, along with potentially a higher degree of dominance compared to mesotrione resistance, suggests that the atrazine resistance trait would be more easily selected under field conditions with normal herbicide use rates (i.e., 1000 g atrazine ha^{-1}). In contrast, evolution of mesotrione resistance may be more dependent on applications of the herbicide below the recommended rate of 105 g ha^{-1}. Although herbicide efficacy under greenhouse conditions does not necessarily equate to that under field conditions, it is clear in Figure 3.1 that there was greater survival of F_1 plants to atrazine than to mesotrione at or near the field use rates (compare survival at 1000 g atrazine ha^{-1} vs. 100 g mesotrione ha^{-1}).

3.5.2 Inheritance of Atrazine Resistance

Dry weight distributions (Figure 3.2) and chi-square analysis (Table 3.1) of the segregating F_2 and BC_s populations all support a single gene inheritance model for atrazine resistance in MCR. Atrazine resistance in MCR likely is due to increased GST-mediated detoxification of the herbicide (Ma et al. 2013). Increased GST-catalyzed metabolism has been previously documented in velvetleaf (*Abutilon theophrasti*) populations (Gronwald et al. 1989; Gary et al. 1996), and inheritance of resistance in this species also followed that of a single, incompletely dominant gene (Anderson and Gronwald 1987). As previously mentioned, two other waterhemp populations, ACR and SegR, also have NTS atrazine resistance. Inheritance of NTS atrazine resistance has not been reported for ACR, but in SegR it apparently is incompletely dominant and multigenic (Patzoldt et al. 2003). Although we cannot rule out the presence of a second, minor gene contributing to atrazine resistance in MCR, inheritance was distinctly different from that described for SegR, and consistent with a single major gene. Future research to compare in parallel the atrazine resistance inheritance patterns of ACR, MCR and SegR populations may provide further insights into the diversity of NTS atrazine resistance genes and mechanisms in waterhemp.

3.5.3 Inheritance of Mesotrione Resistance

Unlike the single gene inheritance of atrazine resistance in MCR, results indicated that mesotrione resistance in MCR is multigenic. Herbicide resistance with multigenic inheritance previously has been reported in other weed populations (e.g., Faulkner 1974; Neve and Powles 2005; Busi et al. 2011; Han et al. 2014).

Although our study of mesotrione resistance inheritance is confounded by the apparent lack of a starting homogenous R population, our conclusion of multigenic inheritance is still supported. For example, if resistance to mesotrione was controlled by a single gene, then R parent heterozygosity would not explain the range of phenotypes that was observed in the F_1 (i.e, with a single gene model, all F_1 plants from a particular cross will either be uniformly intermediate or segregate 1:1). Furthermore, under a single-gene model, even if the original R parent was heterozygous, each F_1 plant used to make BC_s and F_2 lines was selected for resistance. Again assuming a single gene model, each F_1 used in the subsequent crosses therefore should have been heterozygous at the single resistance locus, thereby keeping valid our segregation analysis of the BC_S and F_2 lines.

Attempts to fit the observed segregation ratios with multiple-gene models obtained limited success. For example, although one could invoke multiple additive genes to account for the high proportion of F_2 survivors in the low-rate study, the high mortality of BC_S plants conflicted with the models. Furthermore, the various assumptions required to correct expected ratios (because of segregation of the F_1 lines) and the numerous potential interactions among multiple loci (Han et al. 2014) would render any conclusions of specific multigene models speculative at best.

Mesotrione resistance in MCR has been attributed to P450-based herbicide detoxification (Ma et al. 2013). Although a P450 gene could be one of the resistance loci, it is also possible that a resistance locus is a gene encoding a transcription factor of the P450 gene. One can envision numerous other loci that could modulate P450 activity, or work independently from or in concert with a P450 to confer mesotrione resistance. The

identification of multigenic inheritance of mesotrione resistance in MCR indicates much more research is needed to fully understand the P450-based herbicide detoxification in this population.

In addition to atrazine and mesotrione resistance, the MCR population also has both target site and NTS resistance to acetolactate synthase (ALS) inhibitors (Hausman et al. 2013; Guo et al. 2015). The genetic control of NTS ALS-inhibitor resistance is currently unknown in MCR. It is also unknown whether there is any overlap among the mechanisms and/or genes associated with NTS mesotrione, atrazine, and ALS-inhibitor resistances. One of the concerns of NTS herbicide resistance is that it can lead to unpredictable cross resistance to different herbicide groups (Délye 2013). A better understanding of the specific genes and mechanisms controlling herbicide resistances in the MCR population may provide insights into the evolutionary process by which they were selected.

In summary, this paper adds to the growing body of information on the MCR waterhemp population, which is resistant to herbicides encompassing three sites of action. MCR represents not only a significant weed management threat, but it is an interesting study case for herbicide resistance and evolution. Here we demonstrated that atrazine resistance is mediated by a single major, incompletely dominant, nuclear gene. In contrast, mesotrione resistance in this population is more complex and likely multigenic. The more complex inheritance associated with mesotrione resistance suggests that this trait may spread more slowly, and its evolution may be fostered by the application of reduced herbicide rates.

3.6 Acknowledgements

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3.8 Tables and Figures

Table 3.1: Whole-plant responses to atrazine of resistant (R-NH3) and sensitive parents (S-WCS) and their F_1 progeny.

Population	ED_{50}[1] (g ha^{-1})	R/S[2]
F1-NH5	268.3 (78.6)[3]	7.0 (3.3)
F1-NH6	438.4 (132.9)	11.4 (5.5)
S-WCS	38.4 (14.3)	1
R-NH3	1576.0 (958.2)	41.0 (29)

[1] The effective dose at which plants show a 50% reduction, which was determined using a combination of dry weights and visual observations of herbicide responses.

[2] ED_{50} of resistant or F_1 population by ED_{50} of the sensitive population.

[3] Numbers in parentheses denote ±1 s.e.

Table 3.2: Whole-plant responses to mesotrione of resistant (R-NH3) and sensitive parents (S-WCS) and their F_1 progeny.

Population	ED_{50}[1] (g ha^{-1})	R/S[2]
F_1-NH5	1.5 (0.3)[3]	7.2 (2.3)
F_1-NH6	2.5 (0.4)	4.4 (1.4)
F_1-NH9	2.3 (0.6)	4.8 (1.7)
F_1-NH10	1.4 (0.3)	7.7 (2.7)
S-WCS	0.7 (0.1)	1
R-NH3	10.9 (2.9)	16.3 (4.9)

[1] The effective dose at which plants show a 50% reduction, which was determined using a combination of dry weights and visual observations of herbicide responses.

[2] ED_{50} of resistant or F_1 population divided by ED_{50} of the sensitive population.

[3] Numbers in parentheses denote ±1 s.e.

Table 3.3: Chi-square analysis for goodness of fit of the observed segregation of atrazine resistance in F_2 and BC_s populations. The herbicide rate was chosen, based on previous experiments, to control the sensitive parent but not the resistant parent or F_1 plants. Expected survival is based on a single resistance gene that is dominant at the herbicide rate used.

Run	Rate (g ha^{-1})	Line	No. of plants	Observed survival	Expected survival	χ^2	*p*
1	985	F_2					
		NH51	90	71	68	0.73	0.39
		NH56	84	58	63	1.42	0.23
	985	BC_s					
		NH53	20	5	10	5	0.03
		NH48	24	11	12	0.17	0.68
2	985	F_2					
		NH51	91	63	68	1.61	0.20
		NH56	89	69	67	0.30	0.58
	985	BC_s					
		NH52	28	16	14	0.57	0.45
		NH53	43	16	22	2.81	0.09
		NH48	49	24	25	0.02	0.89

Table 3.4: Chi-square analysis for goodness of fit of the observed segregation of mesotrione resistance in F_2 and BC_s populations. Segregation analysis was performed at high rates of mesotrione, at which resistance was recessive, and a low rate, at which resistance was dominant. Expected survival was based on a single-gene model, and was corrected for the number of survivors observed in parental and F_1 controls that were included in each experimental run.

Run	Rate (g ha^{-1})	Line	No. of plants	Observed survival	Expected survival	χ^2	*p*
Low rate							
1	10	F_2					
		NH51	107	95	59	49.35	<0.001
		NH57	81	66	45	22.95	<0.001
	10	BC_S					
		NH49	48	8	14	4.06	0.04
		NH53	48	7	14	5.43	0.02
2	10	F_2					
		NH50	98	74	42	43.27	<0.001
		NH56	87	65	37	36.57	<0.001
	10	BC_S					
		NH48	43	1	8	6.95	0.01
		NH52	46	2	8	5.60	0.02
3	10	F_2					
		NH50	91	82	59	24.05	<0.001
		NH57	83	75	53	25.85	<0.001
	10	BC_S					
		NH52	32	5	13	7.37	<0.01
		NH48	41	6	17	11.79	<0.01

Table 3.4: (cont.)

Run	Rate (g ha^{-1})	Line	No. of plants	Observed survival	Expected survival	χ^2	*p*
High rate							
1	100	F_2					
		NH50	98	2	25	27.6	<0.001
		NH56	87	3	22	21.6	<0.001
1	120	F_2					
		NH50	98	5	16	9.44	<0.01
		NH56	87	0	15	14.4	<0.001
2	95	F_2					
		NH51	97	5	27	24.1	<0.001
		NH57	72	1	20	24.5	<0.001
2	110	F_2					
		NH51	98	4	21	17.52	<0.001
		NH57	72	1	15	17.17	<0.001

Figure 3.1: (a) Atrazine dose-response curves for atrazine-sensitive population (WCS), atrazine-resistant population (NH3), and F_1 hybrid. The F_1 (R x S) data were pooled from two separate crosses (NH5 and NH6). Vertical bars indicate ±1 s.e. (b) mesotrione dose-response curves for mesotrione-sensitive population (WCS), mesotrione-resistant population (NH3), and reciprocal F_1 hybrids. Each F_1 curve was obtained by pooling two different crosses: NH5 and NH6 for R x S, and NH9 and NH10 for S x R. Vertical bars indicate ±1 s.e.

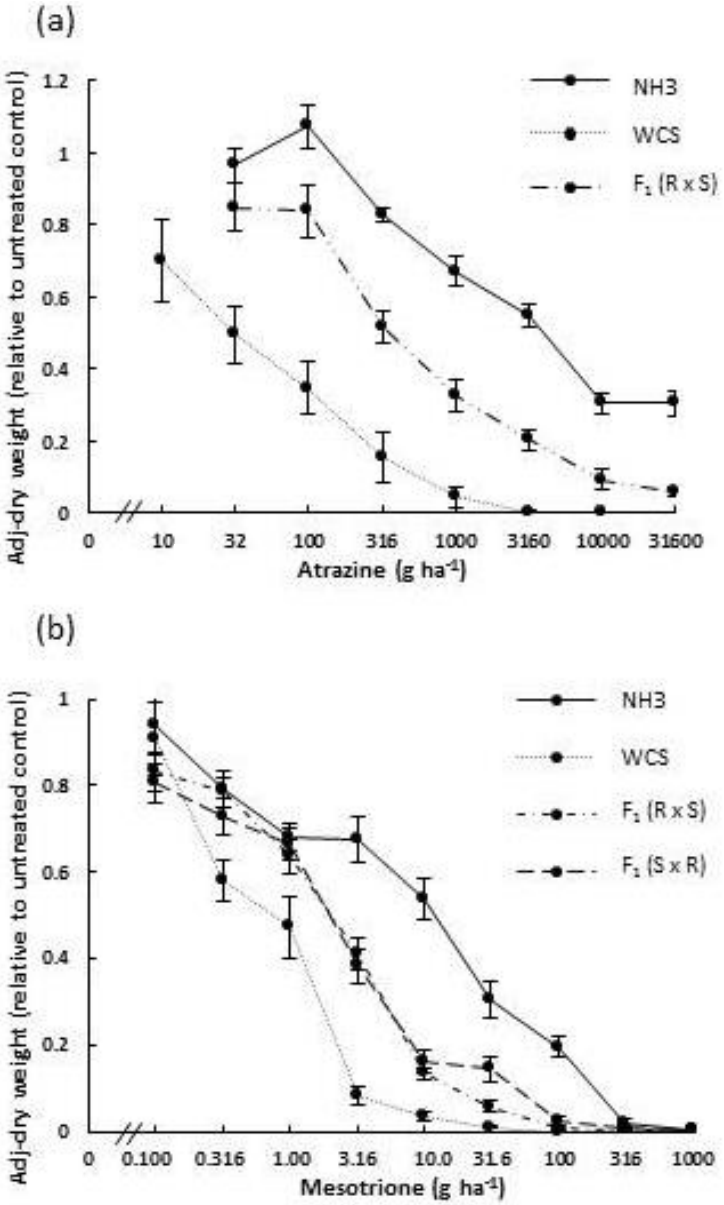
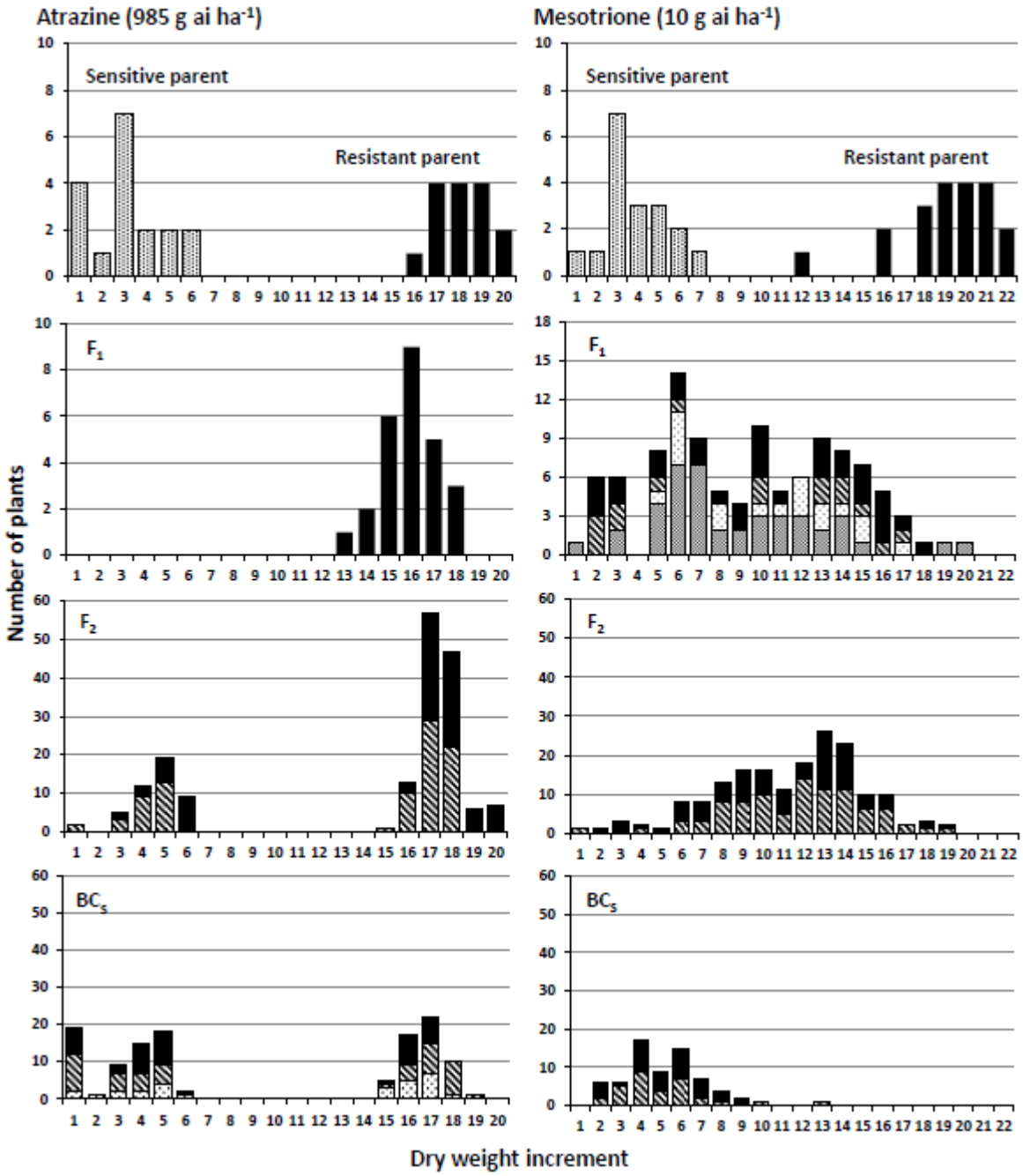


Figure 3.2: Distributions of plant responses to atrazine or mesotrione. Natural-log-transformed dry weights of individual plants were grouped into incremental bins of 0.1. The y-axis indicates the number of plants in a given bin. Multiple lines were pooled for some crosses, with each line represented by a different pattern within the columns.



CHAPTER 4

Concluding Remarks

4.1 Summary and Conclusions

Modern herbicides have significantly contributed to world food production by controlling weeds. With the introduction of herbicide-resistant transgenic crops, an increased dependence on synthetic herbicides for weed control has led to evolutionary growth of herbicide-resistant weed populations. The rate at which resistance evolution occurs depends upon aspects of the herbicide selection (i.e., field history and related agronomic practices), the biology of the weed species undergoing selection (i.e., reproductive type and genetic diversity) and population genetic factors (Jasieniuk et al. 1996). Resistance in weeds occurs by way of two different mechanisms: (1) a direct approach involving a target-site that has been altered or amplified, or (2) an indirect approach involving various methods that minimize the herbicide availability to the target site. The overall purpose of this research was to gain a deeper understanding of TSR and NTSR in weeds.

Prior to determining the mechanism of resistance in the TennGR population, verification of resistance was previously done by Mueller et al. (2011). This population was the first reported case of glyphosate-resistant goosegrass in the United States, and analysis was needed to determine what mechanism(s) could be involved in resistance. Prior studies performed in Tennessee indicated that the resistance factor of the TennGR population was 7.4 (Mueller et al. 2011), but experiments conducted with the same TennGR and TennGS populations at the Plant Care Facility greenhouses (located at the University of Illinois at Champaign-Urbana) indicated that the population had a resistance factor of 3.4. Adjusted-dry weights were used in our study

(factoring in visual observations) whereas Mueller et al. (2011) calculated their resistance magnitude based solely on dry weights. By combining the visual observations with the dry weight data, the regrowth and amount of tillering of each plant were better compared with their corresponding controls. In previous studies where glyphosate resistance was low in magnitude, multiple glyphosate resistance mechanisms were sometimes reported within a single population (Yu et al. 2007; Dinelli et al. 2008; Kaundun et al. 2011; Nandula et al. 2013; Chatham 2014). Based on the research in Chapter 2, it is believed that the TennGR has only one mechanism of glyphosate resistance. This conclusion is supported by two pieces of evidence: (1) Identical resistance levels were observed in the parental populations and the segregating genotypes, and (2) the lack of significant difference in the ED_{50} values of the parental TennGS population (95 g ha^{-1}) and the PP106 genotype (119 g ha^{-1}), or in the ED_{50} values of the parental TennGR population (320 g ha^{-1}) and the SS106 genotype (399 g ha^{-1}). It is interesting to note that ED_{50} valves of the segregating populations are slightly shifted to the right when compared to the parental population, but in spite of slight differences, the populations do not statistically differ.

Regarding the MCR research in Chapter 3, the inheritance of atrazine resistance was controlled by a single gene with incomplete dominance, whereas mesotrione resistance was controlled by multiple genes and can be described as incompletely recessive (Stone 1968). However, the degree of dominance for mesotrione likely underestimates the actual level of dominance because of the lack of homogeneity of the parental R plants and the complicated nature of polygenic traits. Regarding mesotrione, a low level of resistance was seen when analyzing the ED_{50} values, 10.9 ai ha^{-1} for the R population and 1.9 g ai mesotrione ha^{-1} for F_1

population (pooled across F_1 lines herein). It is interesting to note that both R and F_1 population's ED_{50} values were well below normal herbicide use rates, which is 100 g ai mesotrione ha^{-1}. A low level of HPPD resistance observed in the MCR population could be explained by the lack of heterogeneity in the parental lines. Also, selection of the low level of HPPD resistance seen in the MCR population was likely facilitated by reduced herbicide rates that would have allowed for a greater diversity of alleles, including alleles that would not have been selected at a high rate, to accumulate in the population (Délye et al. 2011). HPPD resistance in the MCR population might have taken many years to accumulate enough resistance alleles to be able to produce a noticably resistant population, like in the case of the polygenic inheritance of chlorotoluron resistance in black-grass (Chauvel and Gasquez 1994). In the MCR population, the number of genes responsible for HPPD has yet to be determined. It has not been possible to fit a polygenic model to the observed segregation ratios of the HPPD F_2 and BC_S populations because the high mortality in the BC_S population conflicted with the low mortality in the F_2 population.

4.2 Implications and Future Directions

Goosegrass represents one of the worst weeds in the world (Holm et al. 1977). The occurrence of glyphosate-resistant goosegrass in the United States has brought additional concern for the future of weed management. Before the discovery of the TennGR goosegrass population, the only other reports of glyphosate resistance in goosegrass came from tropical Southeast Asian cropping systems where selection occurred more often due to longer growing

seasons. It is somewhat remarkable that we have created a comparable selection pressure within transgenic crops.

Glyphosate resistance in goosegrass is thought to be a spontaneous event because transfer of resistance traits from other populations are highly unlikely due the species being highly self-pollinated (Ng et al. 2004). So it will be interesting if additional mechanisms, besides a Pro106Ser substitution, were discovered in the glyphosate-resistant goosegrass population from Mississippi (Molin et al. 2013). Only recently, a population of goosegrass was shown to have a very high-level (2,647-fold) in vitro resistance to glyphosate relative to the wild type, and the mechanism of resistance was conferred by a double amino acid substitution in the EPSPS gene (TI102 + PS106 [TIPS]) (Yu et al. 2015). The TIPS mutation in this population of goosegrass recreates the biotechnology-engineered glyphosate-tolerant EPSPS in corn.

A significant amount of research has been accomplished thus far to help facilitate our understanding of the genetics and inheritance of atrazine and HPPD resistances in the MCR population; however, until we have a homogenous HPPD resistant line, we will be unable to analyze how many genes are involved in its resistance. F_3 generations have been made and are currently being tested for homogeneity. Once a uniform HPPD resistant line is made, it will be necessary to create new F_2 and BC_S populations for segregation analysis. It would also be good to compare the genetics and inheritance of the atrazine and HPPD resistances in the MCR population with that of the only other documented case of HPPD resistance found in Palmer amaranth (Sridevi et al. 2014).

In addition to atrazine and mesotrione resistance, the MCR population also has both TS and NTSR to ALS inhibitors (Hausman et al. 2013; Guo et al. 2015). The genetic control of NTS

ALS-inhibitor resistance is not currently known in MCR. It is also unknown whether there is any overlap between the mechanisms and/or genes associated with NTS mesotrione, atrazine, and ALS-inhibitor resistances (Guo et al. 2015). Further studies are underway for analyzing the inheritance of NTSR to ALS inhibitors in the MCR population. F_1, F_2, BC_R and BC_S populations are currently being used in dose response and co-segregation studies. One of the concerns of NTSR is that it may lead to unpredictable cross-resistance to different herbicide groups (Délye 2013). Therefore, a better understanding of the specific genes and mechanisms controlling herbicide resistances in the MCR population may offer insights into the evolutionary process by which they were selected.

This thesis strengthens the need for a more integrated weed management program. Reliance on a single herbicide mode of action was a "cheap and quick fix" for weeds initially, but resulting resistant weeds have caused management to become more costly and time-consuming. In the near future, HPPD inhibitor-resistant crops and synthetic auxin-resistant crops will be marketed, and hopefully our past experiences have taught us a lesson or two in weed management. With resistance mechanisms becoming seemingly more complicated, being able to anticipate evolutionary trajectories in these resistant populations could help prepare a more predictable future for transgenic crops and the herbicides we use in them.

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