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Association between metabolic resistances to atrazine and mesotrione in a multiple-resistant waterhemp (*Amaranthus tuberculatus*) population

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Research Article

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Abstract

Metabolic resistances to atrazine (atz-R) and mesotrione (meso-R) occur in several waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] populations in the United States. Interestingly, although metabolic atz-R but mesotrione-sensitive *A. tuberculatus* populations have been reported, an *Amaranthus* population has not been confirmed as meso-R but atrazine-sensitive, implying an association between these traits. Experiments were designed to investigate whether the single gene conferring metabolic atz-R plays a role in meso-R. An F_2 population was generated from a multiple herbicide–resistant *A. tuberculatus* population from McLean County, IL (MCR). A cross was made between a known meso-R male clone (MCR-6) and a herbicide-sensitive female clone from Wayne County, IL (WCS-2) to develop an F_1 population. Survival of MCR-6 plants following atrazine POST treatment (14.4 kg ha^{-1}) indicated the male parent was homozygous atz-R. F_1 plants were intermated to obtain a segregating pseudo-F_2 population. Dose–response and metabolic studies conducted with mesotrione using F_1 plants indicated intermediate biomass reductions and metabolic rates compared with MCR-6 and WCS. F_2 plants were initially treated with either mesotrione (260 g ha^{-1}) or atrazine (2 kg ha^{-1}) POST, and after 21 d of recovery, vegetative clones from surviving resistant plants were subsequently treated with the other herbicide. When mesotrione was applied first, the meso-R frequency was 8.2%, and when atrazine was applied first, the atz-R frequency was 75%. However, the meso-R frequency increased to 16.5% following preselection for atz-R, and 100% of surviving meso-R plants were atz-R. Our findings indicate that the gene conferring metabolic atz-R is also involved with the meso-R trait within the population tested.

Introduction

Waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] is a small-seeded, summer annual dicot weed that has become increasingly prominent across much of the corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] production areas of the Midwestern United States (Hager et al. 1997; Steckel 2007). *Amaranthus tuberculatus* is indigenous to Illinois but was not a problematic weed species until the widespread adoption of conservation tillage (Refsell and Hartzler 2009) and evolution of multiple herbicide resistances (Tranel et al. 2011), which allowed *A. tuberculatus* to rapidly establish in agronomic production systems (Hager et al. 1997). Management is essential for crop production, because high *A. tuberculatus* densities (8 plants m^{-2}) can reduce soybean and corn yields by up to 56% and 74%, respectively (Steckel 2007).

Amaranthus tuberculatus is capable of high reproductive output in a variety of conditions, with female plants capable of producing more than 400,000 seeds growing in 68% shade to approximately 1 million seeds under ideal conditions (Steckel et al. 2003). These seeds have the ability to remain dormant and viable within the soil seedbank for several years (Buhler and Hartzler 2001; Steckel et al. 2007). In addition to high reproductive output, *A. tuberculatus* is a dioecious and obligate-outcrossing species. Obligate outcrossing promotes high genetic diversity, recombination, and pollen-mediated gene flow in *A. tuberculatus,* which contributes to rapid development of multiple herbicide resistances (Sarangi et al. 2017; Shergill et al. 2018; Tranel et al. 2011). Confirmed resistances to 4-hydroxyphenylpyruvate dioxygenase (HPPD), acetolactate synthase (ALS), protoporphyrinogen oxidase (PPO), photosystem II (PSII), and very-long-chain fatty-acid–inhibiting herbicides, as well as glyphosate and/or the synthetic

auxin 2,4-D, have been reported in several Midwestern *A. tuberculatus* populations (Heap 2020; Shergill et al. 2018; Strom et al. 2019).

In Midwestern corn production, atrazine and HPPD-inhibiting herbicides provide effective chemical options for residual control of *A. tuberculatus* and are typically applied together in PRE or POST tank mixes (Abendroth et al. 2006; Woodyard et al. 2009). Tank mixtures can delay the selection and development of herbicide-resistant weed populations (Evans et al. 2016). Atrazine resistance is commonly conferred by amino acid substitutions in the chloroplast D1 protein, the target site for PSII inhibitors, in resistant weeds (Frenkel et al. 2017). Although target-site and non–target site atrazine-resistance (atz-R) mechanisms exist in weeds (Délye et al. 2013), metabolism-based atz-R is of great concern and has recently become more common (Ma et al. 2013b; Nakka et al. 2017; Preston 2004; Vennapusa et al. 2018; Yu and Powles 2014). The increase in the frequency of metabolic atz-R is potentially due to the lack of a fitness cost typically reported in atz-R weeds with an altered D1 protein (Holt et al. 1993; Vila-Aiub et al. 2009). Metabolic atz-R in a multiple herbicide–resistant (MHR) *A. tuberculatus* population from central Illinois (named MCR for McLean County resistant) is conferred by rapid glutathione (GSH) conjugation to atrazine (Ma et al. 2013b), which is catalyzed by glutathione *S*-transferase (GST) activity (Cummins et al. 2011; Labrou et al. 2015; Perperopoulou et al. 2018).

Mesotrione belongs to the triketone family of HPPD-inhibiting herbicides. HPPD-inhibiting herbicides (Group 27) include the triketones, isoxazoles, and pyrazolones (Beaudegnies et al. 2009; Ndikuryayo et al. 2017). The MCR population (also called the SIR population; O'Brien et al. 2018) described above is both mesotrione-resistant (meso-R) and topramezone-resistant due to rapid oxidative metabolism of the parent herbicide (Lygin et al. 2018; Ma et al. 2013b), presumably catalyzed by one or more cytochrome P450 enzymes (P450s). Greenhouse studies have examined the genetics and inheritance of atz-R and meso-R traits independently in the MCR population (Huffman et al. 2015), and inheritance of meso-R was also investigated in MHR *A. tuberculatus* populations from Iowa (Kohlhase et al. 2018) and Nebraska (Oliveira et al. 2018). Each of these inheritance studies concluded that meso-R in *A. tuberculatus* is a multigenic, or quantitative trait, and Kohlhase et al. (2018) demonstrated that the number of functional genes governing the meso-R trait increased as the mesotrione rate increased. In contrast, metabolic atz-R phenotypically segregated 3:1 (R:S) at 0.98 kg atrazine ha^{-1} POST (Huffman et al. 2015) or 1:2:1 (RR:Rr:rr) at 14.4 kg atrazine ha^{-1} POST (Evans et al. 2017) in a F_2 population derived from MCR, indicating atz-R is inherited as a monogenic, nuclear-encoded trait.

Populations within *A. tuberculatus* and Palmer amaranth (*Amaranthus palmeri* S. Watson), the only two weed species with confirmed HPPD inhibitor–resistant populations, are also multiple resistant to atrazine (Heap 2020). This suggests a possible association between these two traits in spite of presumed distinct detoxification mechanisms (Evans et al. 2017; Ma et al., 2013b). By contrast, atz-R *A. tuberculatus* populations that remain sensitive to mesotrione have been commonly reported (Heap 2020; Patzoldt et al. 2005; Yu and Powles 2014). It is possible, however, that the single gene conferring metabolic atz-R (Huffman et al. 2015) might be one of several unknown genes involved in meso-R in the MHR *A. tuberculatus* populations. In the current study, experiments were designed to investigate the hypothesis that metabolic atz-R and meso-R traits were independently controlled in a segregating F_2 population. The following objectives were designed to experimentally test our hypothesis: (1) generate a pseudo-F_2 population that segregates for meso-R and atz-R traits, and (2) develop a screening procedure that allows surviving meso-R F_2 plants to be subsequently treated with atrazine and phenotypically assessed for atz-R, and vice versa.

Materials and Methods

Generation of F_1 and Pseudo-F_2 Populations, Dose Response, and Metabolism of Mesotrione

Greenhouse Crosses

Based on our previous results regarding mesotrione metabolism in the MCR population (Ma et al. 2013b), a single cross was made between one male (MCR-6) parent and one female (WCS-2) parent to generate an F_1 (Ma et al. 2013a). The MCR-6 clonal line was selected because it displayed the fastest rate of mesotrione metabolism among the six meso-R lines tested, while the WCS-2 line displayed one of the slowest mesotrione metabolic rates among sensitive lines (Ma et al. 2013b). Five male F_1 plants were then intermated with a single female F_1 plant to create a pseudo-F_2 population. Although the exact number of genes conferring meso-R is currently unknown (Huffman et al. 2015; Kohlhase et al. 2018), the MCR-6 male clone was chosen for making a cross with the sensitive WCS-2 parent due to the greater likelihood of MCR-6 possessing a homozygous meso-R genotype at each locus based on its rapid mesotrione metabolic rate (Ma et al. 2013b). By contrast, male MCR parents chosen for crosses in prior inheritance work (Huffman et al. 2015) likely included some heterozygous individuals.

Mesotrione Dose–Response Analysis

A whole-plant dose–response study was conducted with the MCR-6 clonal line, WCS population (the WCS-2 clonal line was lost during continuous vegetative cloning), and the F_1 under similar greenhouse and plant culturing conditions as described previously (O'Brien et al. 2018). All seeds were stratified using methods outlined by Bell et al. (2013), then placed in 12-cm potting tray inserts filled with commercial potting soil (LC1, Sun Gro Horticulture, 15831 NE 8th Street, Bellevue, WA 98008). Seeds were germinated in a growth chamber with 28/22 C day/night temperatures and a photoperiod of 16/8 h. Light within the growth chamber was provided by incandescent and fluorescent bulbs that delivered 550 µmol m^{-2} s^{-1} photon flux at the plant canopy. Plants were then transferred to the greenhouse, and upon reaching 3 to 4 cm were evenly spaced within a heavy-duty planter flat in LC1 potting soil supplemented with slow-release fertilizer (Osmocote® 13-13-13, Scotts, 14111 Scottslawn Road, Marysville, OH 43041). Natural sunlight in the greenhouse was supplemented with mercury halide lamps to provide 800 µmol m^{-2} s^{-1} photon flux at the plant canopy level as previously described (O'Brien et al. 2018).

Mesotrione (Callisto™ herbicide, Syngenta Crop Protection, Greensboro, NC 27409) was applied POST to 10- to 12-cm plants with 1% (v/v) crop oil concentrate (COC) (Herbimax®, Loveland Products, Loveland, CO 80538) and 2.5% (v/v) liquid ammonium sulfate (AMS) (N-Pak®, Winfield United, Shoreview, MN 55126) as adjuvants. Mesotrione rates ranged from 0.105 to 10,500 g ha^{-1}, spaced equally on a log 3.16 scale (105 g ha^{-1} is the labeled POST rate in corn). Treatments were applied with an automated compressed-air research sprayer (DeVries Manufacturing, 86956 State Highway 251, Hollandale, MN 56045) fitted with

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a TeeJet® 80015 EVS nozzle (TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187) calibrated to deliver 185 L ha^{-1} at 275 kPa at 45 cm above the plant canopy. Aboveground biomass was harvested at 14 d after treatment (DAT) and dried in an oven at 65C for 7 d. Dry weights were then recorded and converted into a percentage of the respective nontreated control plants.

Dry-weight data were collected from two separate studies, with treatments arranged in a completely randomized design with six replications. Levene's test for homogeneity of variance was not significant; as a result, the data from both studies (12 replications in total) were pooled and analyzed by nonlinear regression using the dose–response curve package in R (Knezevic et al. 2007). Rates of mesotrione required to decrease plant biomass by 20%, 50%, and 80% (GR_{20}, GR_{50}, and GR_{80}, respectively) were determined as previously described (Ma et al. 2016; O'Brien et al. 2018) using the following four-parameter nonlinear logistic model (Equation 1).

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

where b is the slope of the curve, c is the lower limit, d is the upper limit, and GR_{50} is 50% reduction in dry weight.

Mesotrione Metabolism Using an Excised Leaf Assay

Rates of mesotrione metabolism were measured by the time required for 50% of absorbed mesotrione to degrade (DT_{50}) during a time-course study using the same plant materials described earlier. Excised leaves from plants (10 to 12 cm) from each line or population (MCR-6, WCS, and F_1) were incubated for 1 h in a 0.1 M Tris-buffered solution (pH 6.0) containing 150 µM [URL-^{14}C] mesotrione in a growth chamber, as described previously by Ma et al. (2013b, 2015). Two independent experiments arranged in a completely randomized design were conducted for the metabolic study, and each treatment (line/population by time point) was replicated six times. Levene's test for homogeneity of variance was not significant; as a result, the data from both studies (12 replications in total) were pooled and analyzed by nonlinear least-squares regression analysis and fit with a simple first-order curve in order to estimate DT_{50} values. The model was described by the following equation.

$$y = C_0 e^{-\frac{\ln(2)}{\mu_i}t} \quad [2]$$

where y represents the percentage of the parent herbicide remaining at time t, μ_i is the DT_{50} for each biotype i, and the parameter C_0 is the estimated amount of parent herbicide present at $t = 0$.

Characterization of Atrazine Resistance in the Male MCR-6 Parent

The MCR-6 × WCS-2 cross (described previously in "Methods") was performed with the intent of investigating the genetics and inheritance of the meso-R trait (Ma et al. 2013a), but the phenotype of the MCR-6 clonal line in regard to atz-R was not determined before the cross was made. Before the F_2 population could be used for experiments to investigate our hypothesis that meso-R and atz-R traits in *A. tuberculatus* are independently controlled, it was necessary to determine whether the male MCR-6 parent was homozygous atz-R. A discriminatory atrazine POST rate was used to distinguish between the two possible atz-R genotypes (RR and Rr) based on their distinct phenotypes (Evans et al. 2017). MCR-6 vegetative clones were continually propagated in a growth chamber and greenhouse, as described previously (Ma et al. 2013b, 2015) under the same growth conditions described earlier. Once the cloned MCR-6 plants had reached a height of 9 to 11 cm (at or before the 8-leaf stage), atrazine was applied POST at a rate of 14.4 kg ha^{-1} (with 1% [v/v] COC and 2.5% [v/v] AMS), and treated plants were then returned to the greenhouse. *Amaranthus tuberculatus* injury was assessed by visual inspection at 7, 14, and 21 DAT based on the amount of chlorotic and necrotic tissue and height differences. Plants that rapidly developed healthy, new green tissue following atrazine treatment and were as tall as nontreated controls were assigned a homozygous (RR) atrazine-R genotype (Figure 1A–C). By comparison, plants that developed less green meristematic tissue than RR lines following atrazine treatment, were stunted, and did not grow significantly taller after application were considered representative of the heterozygous (Rr) atrazine-R genotype (Figure 1D). Plants that died at the 14.4 kg ha^{-1} discriminatory rate within 7 DAT were assigned an atrazine-sensitive (rr) genotype (Figure 1E) (Evans et al. 2017).

Sequential Treatments and Segregation Analysis of the Pseudo-F_2 Population

Plants from the previously described F_2 population at a height of 9 to 11 cm were treated with either 260 g ha^{-1} mesotrione (including 1% [v/v] methylated seed oil [MSO] [MSO concentrate, Loveland Products]) or 2 kg ha^{-1} atrazine (with 1% [v/v] COC and 2.5% [v/v] AMS). The 2 kg ha^{-1} atrazine rate was chosen because it represents the maximum labeled POST rate in corn and clearly distinguishes atz-R from atrazine-sensitive plants; that is, resistance could be identified visually, because homozygous and heterozygous atz-R F_2 plants exhibited little injury and regrew rapidly, while sensitive plants quickly died (Evans et al. 2017; Huffman et al. 2015). The relatively high rate of POST mesotrione was chosen based on preliminary tests to differentiate meso-R from mesotrione-sensitive plants but without killing intermediately meso-R individuals (described in detail below). The rate of mesotrione POST (260 g ha^{-1}) also represents an approximate GR_{80} rate determined in previous greenhouse studies by our research group with the MCR/SIR population (Hausman et al. 2011; O'Brien et al. 2018) that ensured sensitive plants would not survive.

Following the first mesotrione treatment, assessments of visual injury were recorded 7, 14, and 21 DAT on a scale of 1 to 10. A rating of 1 represented minimal injury (mainly green tissue), while a rating of 10 represented complete plant death by 21 DAT. Due to the polygenic nature of meso-R in A. *tuberculatus* (Huffman et al. 2015), a 1 to 10 rating scale was, chosen because a range of phenotypes were observed (in accord with Kohlhase et al. 2018). Using the 1 to 10 scale (Figure 2), plants with ratings of 1 to 5 at 21 DAT were considered meso-R. A rating of 1 or 2 reflected a plant with minimal bleaching and necrosis (possibly homozygous meso-R at each locus; Figure 2A and B). A rating of 3 to 5 reflected plants with a completely bleached apical meristem that retained some green leaf tissue on the lower part of the plants. Plants with bleached meristems that were rated 3 to 5 survived to 21 DAT, and eventually reached reproductive stages (Figure 1C and D, plants with a rating of 3 are shown). Plants rated 6 to 10 incurred significant initial injury through 21 DAT and died before flowering (Figure 2E–H).

Because a range of meso-R phenotypes were present in the segregating F_2 population (Figure 2), some meso-R plants incurred significant initial bleaching and necrosis at the apical meristem and axillary buds, which made the vegetative cloning process

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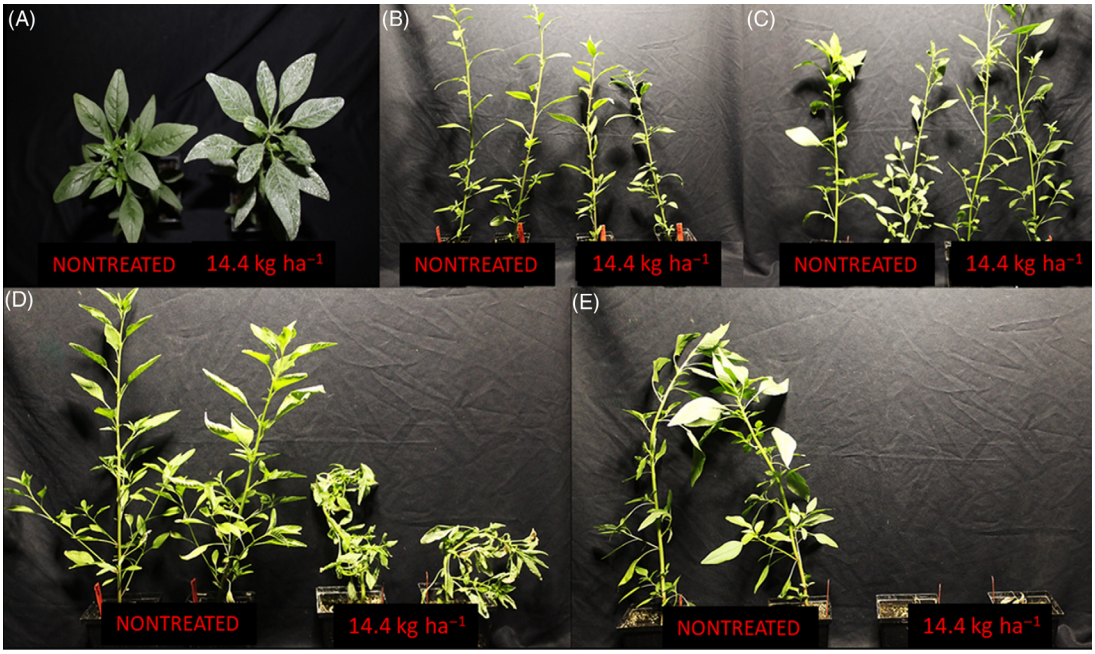

Figure 1. Phenotypic segregation of MCR-6 (cloned male parent) and individual F_2 clonal lines of *Amaranthus tuberculatus* in response to foliar-applied atrazine at 14 d after treatment. MCR-6 and several F_2 plants (9 to 11 cm) were treated with 14.4 kg ha^{-1} atrazine (with 1% [v/v] crop oil concentrate and 2.5% [v/v] liquid ammonium sulfate) to distinguish among the three possible atrazine-resistant (atz-R) phenotypes (described in Evans et al. 2017). (A) Top view of MCR-6 nontreated and treated plants showing lack of foliar injury and necrosis; (B) side view of MCR-6 nontreated and treated plants showing lack of foliar injury, necrosis, and minimal height reduction; (C) side view of homozygous atz-R F_2 plants; (D) side view of heterozygous atz-R F_2 plants; and (E) side view of homozygous atrazine-sensitive F_2 plants. The four plants in each of panels C to E were cloned from a single, randomly chosen F_2 "mother" plant, and thus are genetically identical.

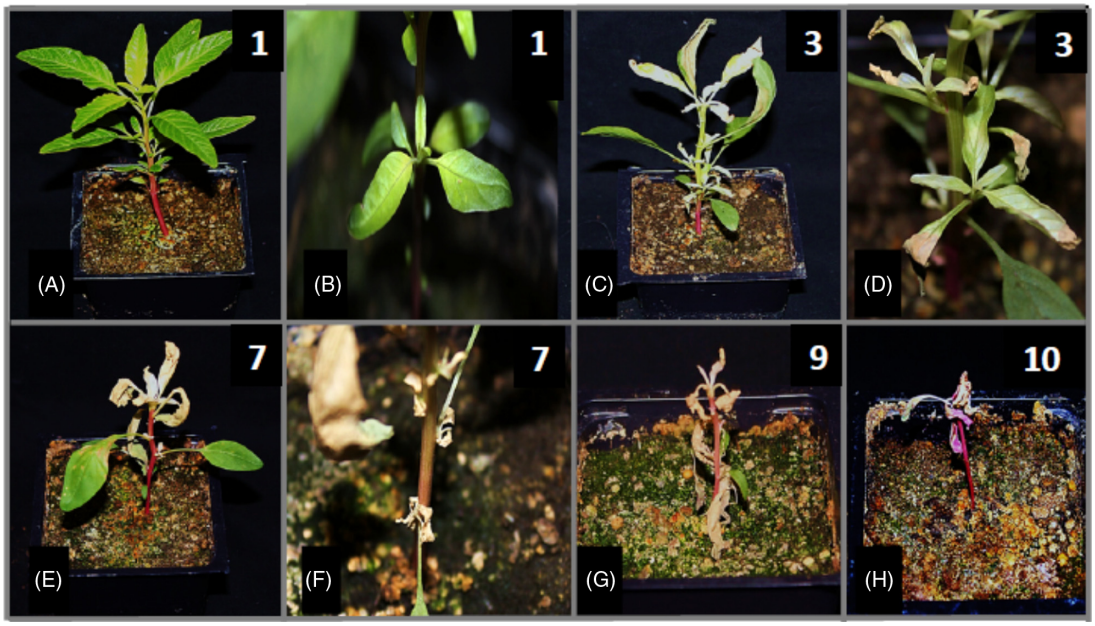

Figure 2. Representative visual assessment of injury scale illustrating the 1 to 10 ratings used to characterize the range of symptomology exhibited by mesotrione-treated F_2 plants (white number in upper-right corner of each panel). A mesotrione-resistant (meso-R) F_2 plant showing minor bleaching symptomology, representing an injury rating of 1, shown at the seedling level (A) and close-up of an axillary bud (B). A meso-R F_2 plant displaying more bleaching and some necrosis on leaf tips but eventually recovered, representing an injury rating of 3, shown at the seedling level (C) and close-up of axillary buds (D). A mesotrione-sensitive F_2 plant demonstrating severe necrosis that killed the meristems; the plant eventually died before 21 d after treatment (DAT), representing an injury rating of 7, shown at the seedling level (E) and close-up of axillary buds (F). Mesotrione-sensitive F_2 plants that died within 14 DAT, representing injury ratings of 9 and 10 (G, H).

extremely difficult (particularly for plants rated 4 or 5). A foliar antioxidant solution was developed and tested to overcome the initial oxidative injury generated by mesotrione (Hess 2000; Mitchell et al. 2001) and "rescue" intermediately meso-R plants. The solution consisted of 0.1 mM sodium ascorbate (final pH adjusted to 6.0 with 0.1 N HCl), 0.1 mM vitamin A acetate (diluted from a stock in 95% dimethyl sulfoxide), and 20 µM Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic

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acid; Sigma-Aldrich, St Louis, MO 63103) diluted from a stock in absolute ethanol. Trolox is a water-soluble, synthetic form of vitamin E that has been used previously to scavenge free radicals in biological systems (Hamad et al. 2010). The spray solution also included 0.25% (v/v) nonionic surfactant (Activator 90, Loveland Products) to facilitate spray absorption and leaf wetting and 1% (v/v) ethanol to increase compound solubility. The antioxidant solution was applied liberally with a hand mist sprayer at 14 DAT with mesotrione, then again at 21 DAT (just before vegetative cloning; described below) to facilitate recovery of intermediately meso-R plants (plants rated 3 to 5; Figure 2). Solutions were prepared the day of application to avoid oxidation and instability of antioxidants. In preliminary tests to investigate the potential of an indirect safening response by the antioxidant solution, 10- to 12-cm atrazine-sensitive plants not treated with either herbicide were treated with the antioxidant solution and then again at 7 d after antioxidant application. Plants were then treated at 14 d after the initial antioxidant application with 2 kg ha^{-1} atrazine and assessed visually for injury and height differences. Repeated foliar applications of the antioxidant solution did not induce atz-R in *A. tuberculatus* plants.

The apical meristems from individual meso-R or atz-R F_2 plants from the first herbicide treatment were clipped at 21 DAT to facilitate axillary bud growth and cloning (Ma et al. 2015). Cloning resistant survivors was necessary in order to apply the second herbicide treatment to 9- to 11-cm-tall plants, as the original resistant parent plants continued to grow during the 21-d recovery period and resulted in plants outside labeled heights for control. Axillary buds from each resistant plant were placed in individual pots within plastic inserts filled with LC1 potting soil and returned to the growth chamber for 4 to 5 d to establish roots. To promote root growth, the axillary buds were treated with a powder containing indole-3-butyric acid (Sigma-Aldrich). Seedlings were transferred to the greenhouse until the second POST herbicide treatment, as described earlier.

To investigate the potential genetic relationship between the two resistance traits in the F_2 population, the same screening procedure was conducted, but in the reverse order, following the first herbicide treatment. For example, surviving atz-R plants were treated with 260 g ha^{-1} mesotrione, and meso-R plants were treated with 2 kg ha^{-1} atrazine POST when plants reached 9 to 11 cm in height. Each experiment was repeated 10 times (Trials 1 to 10 or 11 to 20) with 20 to 89 individual F_2 plants tested within each trial for the first herbicide treatment. To ensure the percentage of atz-R and meso-R plants was consistent in both studies, regardless of the order in which the herbicides were applied, the probabilities of these intersections were compared using a combination of conditional probability and χ^2 goodness-of-fit tests ($\alpha = 0.05$; Bernardo 2010; Ott and Longnecker 2010). Specifically:

$$P(A \cap M) = P(A|M) \times P(M) \quad [3]$$

where $P(A \cap M)$ is the probability that a plant is both atz-R and meso-R; $P(A \mid M)$ is the conditional probability of atz-R, given that a plant is meso-R; and $P(M)$ is the marginal probability of meso-R. In the inverse study, the following equation was used:

$$P(M \cap A) = P(M|A) \times P(A) \quad [4]$$

where $P(M \cap A)$ is the probability that a plant is both meso-R and atz-R; $P(M \mid A)$ is the conditional probability of meso-R, given that a plant is atz-R; and $P(A)$ is the marginal probability of atz-R.

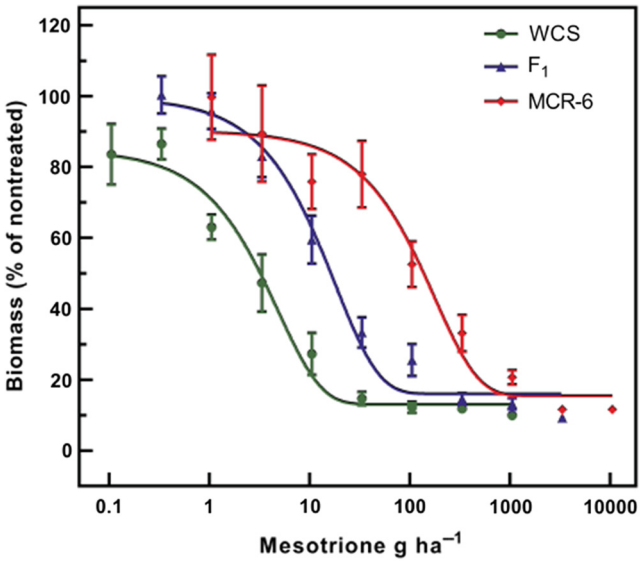

Figure 3. Mesotrione dose–response curves for the male parent (MCR-6) clonal line, WCS population, and the F_1 generated by crossing MCR-6 with WCS-2. Mesotrione was applied to 10- to 12-cm plants at rates spaced on a log 3.16 scale ranging from 0.105 to 10,500 g ha^{-1} with 1% (v/v) crop oil concentrate and 2.5% (v/v) liquid ammonium sulfate as adjuvants. Two independent experiments were conducted with six replications each. Dry-weight data were pooled and analyzed by nonlinear least-squares regression using the dose–response curve package in R (Knezevic et al. 2007) to determine rates of mesotrione required to decrease plant biomass by 20%, 50%, and 80% (GR_{20}, GR_{50}, and GR_{80}, respectively), as previously described (Ma et al. 2016; O'Brien et al. 2018). Vertical bars represent the treatment mean ± SE.

Provided the order in which the herbicide is applied does not influence the likelihood of a plant being both atz-R and meso-R, $P(A \cap M)$ and $P(M \cap A)$ should be nonsignificantly different, as verified by a χ^2 goodness-of-fit test.

Results and Discussion

Generation of F_1 and Pseudo-F_2 Populations, Dose Response, and Metabolism of Mesotrione

Mesotrione Dose–Response Analysis

F_1 plants generally displayed an intermediately meso-R phenotype (e.g., Figure 2C and D) in relation to the MCR-6 parent and WCS (Figure 3). Resistance indices (RIs) of 36-fold, 50-fold, and 66-fold were calculated based on GR_{20}, GR_{50}, and GR_{80} values, respectively, for MCR-6 relative to WCS following mesotrione treatment (Table 1). Both the GR_{50} (105 g ha^{-1}) and RI (50-fold) for MCR-6 are greater than the GR_{50} (48.5 g ha^{-1}) and RI (35-fold) calculated for the MCR field population in relation to WCS in previous research (Hausman et al. 2011). The greater GR_{50} and RI of the MCR-6 clone (relative to WCS; Table 1) compared with the original MCR population (also calculated relative to WCS; Hausman et al. 2011) supports a greater likelihood for homozygosity at one or more genetic loci conferring meso-R in the MCR-6 male clone compared with the MCR plants used in previous genetics and inheritance research (Huffman et al. 2015). However, further molecular–genetic studies are required to verify the actual meso-R genotype of the MCR-6 male clone.

Mesotrione Metabolism Using an Excised Leaf Assay

Rapid mesotrione metabolism by the MCR-6 clone (DT_{50} of 10.6 h) and the inability of WCS to reach a DT_{50} (>36 h) was

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Table 1. Pooled data from the mesotrione dose–response experiment (Figure 3).[a]

Population[b]	GR_{20}	GR_{50}	GR_{80}
WCS	0.4 (± 0.2)	2.1 (± 0.6)	11.7 (± 5.0)
F_1	3.0 (± 1.1)	12.2 (± 2.8)	48.7 (± 19.0)
MCR-6	14.3 (± 7.3)	105 (± 35.3)	767 (± 473)

[a]Estimated GR_{20}, GR_{50}, and GR_{80} values (rates of mesotrione required to decrease plant biomass by 20%, 50%, and 80%, respectively) are expressed as rates of mesotrione in g ha^{-1}. GR values are followed by their respective standard errors of the mean in parentheses.
[b]*Amaranthus tuberculatus* populations: MCR-6, cloned male parent; WCS, herbicide-sensitive population; F_1, generated by crossing MCR-6 with WCS-2.

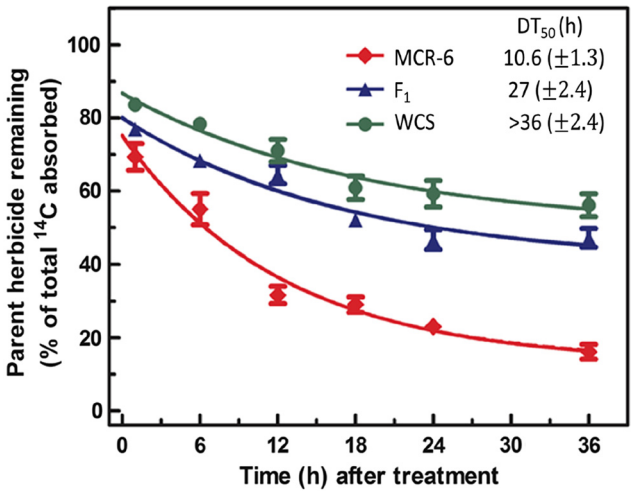

Figure 4. Mesotrione metabolism in excised *Amaranthus tuberculatus* leaves from the male parent (MCR-6) clonal line, WCS population, and the F_1 generated by crossing MCR-6 with WCS-2. Excised leaves from 10- to 12-cm plants were treated with 150 µM [URL-^{14}C]mesotrione for 1 h, and a time-course study was conducted to determine the time required for 50% of absorbed mesotrione to degrade (DT_{50}), as described by Ma et al. (2013b, 2015). Two independent experiments were conducted with six replications each. Data were pooled, analyzed by nonlinear least-squares regression analysis, and fit with a simple first-order curve to estimate DT_{50} values. Vertical bars represent the treatment mean ± SE, and DT_{50} values for each line or population are shown ±SE.

measured (Figure 4), which is in accord with previous research analyzing the MCR-6 clone (DT_{50} of 9.5 h) and several WCS clones (median DT_{50} of >45 h) (Ma et al. 2013b). Additionally, the F_1 displayed an intermediate DT_{50} (27 h) that was closer to WCS in the mesotrione metabolic study than MCR-6, particularly at the later time points examined (Figure 4). The metabolic results are consistent with GR_{50} values determined for POST mesotrione in the dose–response study (Table 1; Figure 3). When taken together with the dose–response results (Figure 3) that indicated greater fold resistance of the MCR-6 clone relative to the MCR population (Hausman et al. 2011), the mesotrione metabolic results rationalize our selection of the MCR-6 and WCS-2 clones to generate F_1 and pseudo-F_2 populations for meso-R segregation analysis.

Characterization of Atrazine Resistance in the Male MCR-6 Parent

Typical foliar necrosis symptoms resulting from atrazine (14.4 kg ha^{-1}) were not detected in any treated MCR-6 cloned plant (Figure 1A and B). In addition, significant height reductions were not observed in any treated MCR-6 plant in comparison to nontreated control plants at 7, 14, and 21 DAT (Figure 1B, only 14 DAT is shown). The lack of injury to or significant height

Table 2. Sequential phenotyping experiment in a segregating F_2 population to quantify resistant individuals to mesotrione and atrazine.

Trial number	Number of plants treated	First treatment[a]	% Resistant[b]	Second treatment[a]	% Resistant[c]
1	76	Mesotrione	7.9	Atrazine	100
2	81	Mesotrione	14.8	Atrazine	100
3	81	Mesotrione	4.9	Atrazine	100
4	73	Mesotrione	8.2	Atrazine	100
5	77	Mesotrione	6.5	Atrazine	100
6	89	Mesotrione	10.1	Atrazine	100
7	88	Mesotrione	15.9	Atrazine	100
8	63	Mesotrione	4.8	Atrazine	100
9	56	Mesotrione	1.8	Atrazine	100
10	58	Mesotrione	6.9	Atrazine	100
11	20	Atrazine	70	Mesotrione	21.4
12	24	Atrazine	75	Mesotrione	22.2
13	26	Atrazine	76.9	Mesotrione	15
14	30	Atrazine	70	Mesotrione	19.1
15	30	Atrazine	80	Mesotrione	8.3
16	35	Atrazine	80	Mesotrione	7.1
17	30	Atrazine	76.7	Mesotrione	17.4
18	30	Atrazine	76.7	Mesotrione	13
19	30	Atrazine	73.3	Mesotrione	22.7
20	30	Atrazine	70	Mesotrione	19.1

[a]Mesotrione was applied at 260 g ha^{-1} with 1% (v/v) methylated seed oil. Atrazine was applied at 2 kg ha^{-1} with 1% (v/v) crop oil concentrate and 2.5% (v/v) liquid ammonium sulfate as adjuvants.
[b]Original F_2 plants treated with foliar herbicide (mesotrione or atrazine).
[c]Vegetatively cloned plants, derived from surviving resistant plants from the first herbicide treatment, were treated with the other herbicide when plants reached a height of 9 to 11 cm as described in "Methods."

reductions in treated plants indicates that the cloned MCR-6 plants, and thus the original MCR-6 male parent, are homozygous atz-R. To further investigate the genotype of MCR-6, several randomly chosen plants were selected from the segregating F_2 population and treated with the same discriminatory atrazine rate to compare with the phenotype of MCR-6 as described previously (Evans et al. 2017). As expected, three distinct phenotypes were observed, and representative F_2 plants are shown (Figure 1C–E) to illustrate typical plant responses within each genotypic category. Importantly, homozygous atz-R F_2 plants (Figure 1C) were phenotypically similar to MCR-6 (Figure 1B) and clearly distinguishable from heterozygous atz-R F_2 plants (Figure 1D).

Sequential Treatments and Segregation Analysis of the Pseudo-F_2 Population

When mesotrione was applied POST first at 260 g ha^{-1}, a range of meso-R phenotypes were delineated by using the portion of the rating scale from 1 to 5 describing meso-R plants (details listed in "Methods" and representative ratings of 1 and 3 shown in Figure 2A–D). The overall treatment mean percentage of meso-R plants was 8.2% ($P(M) = 8.2\%$; Table 2), which is consistent with a quantitative, polygenic trait as well as previous reports describing meso-R in *A. tuberculatus* (Huffman et al. 2015; Kohlhase et al. 2018). Atrazine treatment at a rate of 2 kg ha^{-1} clearly separated atz-R (both homozygous and heterozygous) plants from atrazine-sensitive plants under greenhouse conditions (data not shown). Surprisingly, 100% of all meso-R clones were atz-R (Table 2), empirically determining the inheritance of meso-R is genetically related to inheritance of atz-R. The conditional probability ($P(A \mid M) = 100\%$) strongly supports that either the gene conferring atz-R is also involved with controlling meso-R or that the genes are linked. To determine which of these two possibilities was more plausible,

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the marginal and conditional probabilities of the inverse study were also calculated (Equations 3 and 4).

In the inverse study, the overall treatment mean percentage of atz-R plants (easily discernible by 14 DAT) where atrazine was applied first was 74.9% (Table 2). Atz-R plants were then cloned as previously described and treated with mesotrione POST. The observed frequency of plants that were both meso-R and atz-R in the inverse study was 12.4% (Equation 4). Using a χ^2 goodness-of-fit test, this percentage (i.e., the probability that a plant is resistant to both herbicides) was found to be nonsignificantly different from the observed 8.2% across the 10 trials (P-value = 0.30), as expected. Therefore, the order in which the herbicides were applied did not influence the probability that a plant was resistant to both herbicides. Furthermore, the conditional probability that plants were meso-R given they were atz-R ($P(M \mid A)$) was experimentally determined as 16.5% (Table 2), which is significantly greater than the observed frequency of mesotrione resistance without atrazine prescreening (8.2%) (P-value = 0.006). Comparing conditional probability results from the atrazine first–mesotrione second study ($P(M \mid A) = 16.5\%$) to the conditional probability determined in the complementary study ($P(A \mid M) = 100\%$) strongly supports the hypothesis that the gene conferring metabolic atz-R is also involved with controlling the meso-R trait, but that additional gene(s) are necessary to confer meso-R in MCR-6.

Interestingly, the conditional probability $P(M \mid A) = 16.5\%$ (atrazine first–mesotrione second experiment) calculated in our research is consistent with a non-epistatic, independently assorting model that includes a sole atz-R locus and two additional loci that segregate in a ratio of 13:3 (S:R). These results are also consistent with a recently proposed model for mesotrione resistance in *A. tuberculatus* from Iowa (Kohlhase et al. 2018), where greenhouse results showed that three genetic loci governed resistance at mesotrione rates ≥210 g ai ha^{-1}. Importantly, the rate of mesotrione used for screening in our greenhouse experiments was 260 g ha^{-1} (Table 2). However, more detailed research studies are necessary to further refine and test this hypothesis and genetic model experimentally.

In the case of mesotrione resistance and atrazine resistance in MCR-6, our results imply that 100% of meso-R plants in the F_2 population are also atz-R, but atz-R plants are not necessarily meso-R. This theory can be explained if the single gene in MCR-6 conferring atz-R (most likely encoding a GST that rapidly metabolizes atrazine; Evans et al. 2017) is necessary, but not sufficient by itself to confer mesotrione resistance, which likely requires at least two additional genes at mesotrione rates ≥210 g ai ha^{-1} (in accord with Kohlhase et al. 2018). The contribution of a qualitative trait contributing to a different quantitative trait has been previously hypothesized for plant pathogen resistance (Nelson et al. 2018). Our proposed genetic model is consistent with the increase in meso-R frequency quantified following preselection for atz-R (8.2% to 16.5%), coupled with the inverse experiment showing an increase in atz-R frequency from 75% to 100% following preselection for meso-R (Table 2). Our determination of an association between meso-R and atz-R traits is also indirectly supported by several reports of meso-R and metabolic atz-R *A. tuberculatus* plants and populations in the field (Heap 2020), where all known meso-R *A. tuberculatus* populations are also atz-R (Heap 2020; Ma et al. 2013b). However, several reported atz-R *A. tuberculatus* populations (due to rapid metabolism) are not meso-R; for example, the Adams County, IL, resistant population that possesses metabolic atz-R (Ma et al. 2013b) is sensitive to mesotrione and other HPPD-inhibiting herbicides (O'Brien et al. 2018; Patzoldt et al. 2005). The F_2 plants treated with antioxidant solution before the application of atrazine also followed a 3:1 (R:S) segregation ratio (data not shown), indicating that this solution did not confer an indirect safening response that would interfere with the data analysis from the other screenings in our study.

Mesotrione metabolism in corn is due to P450-catalyzed hydroxylation of the dione ring (Hawkes et al. 2001), and a similar P450-catalyzed pathway (evidenced by formation of 4-hydroxy-mesotrione) rapidly metabolized mesotrione in the MCR population (Ma et al. 2013b). More recent research has demonstrated the rice (*Oryza sativa* L.) gene *HIS1*, which encodes a Fe(II)/2-oxoglutarate–dependent oxygenase enzyme, governs tolerance to several triketone HPPD-inhibiting herbicides in rice via oxidative metabolism (Maeda et al. 2019). Because GSH conjugates of mesotrione have not been identified in crops or weeds to date (Hawkes et al. 2001; Kaundun et al. 2017; Ma et al. 2013b; Mitchell et al. 2001), it might seem implausible to suggest a GST enzyme could be associated with the meso-R trait in *A. tuberculatus*. However, some GST proteins catalyze glutathione peroxidase (GPOX) or dehydroascorbate reductase activity, where GSH becomes oxidized and cytotoxic lipid peroxides or dehydroascorbate, respectively, are reduced during each reaction (Mashiyama et al. 2014; Perperopoulou et al. 2018). These reactions differ from GST-catalyzed conjugation of GSH directly to herbicide substrates (e.g., atrazine, chloroacetamides) possessing an electrophilic site (Riechers et al. 2010), because GSH is consumed (Cummins et al. 2011). The "indirect" mechanism for hydroperoxide detoxification by a GST with GPOX activity (*Am*GSTF1) has been reported in MHR blackgrass (*Alopecurus myosuroides* Huds.) from the United Kingdom, where resistances to several classes of herbicides that generate oxidative stress was reported, such as the phenylureas (Cummins et al. 1999, 2013). Accordingly, we propose the same GST enzyme that metabolizes atrazine in MCR-6 also contributes to meso-R by detoxifying lipid peroxides resulting from triplet chlorophyll and reactive oxygen species that are produced following mesotrione treatment (Beaudegnies et al. 2009; Hess 2000; Mitchell et al. 2001). Our theory is further substantiated by research in rice with an overexpressed GST (*Os*GSTL1) possessing GPOX activity, in which transgenic plants displayed enhanced herbicide tolerance (Hu et al. 2009). Moreover, some enzymes and proteins demonstrate multifunctional or "moonlighting" activities (Huberts and van der Klei 2010; Jeffery 2014; Ke et al. 2020) that might also occur with certain *A. tuberculatus* GSTs. A multifunctional enzyme is somewhat analogous to a single gene conferring metabolic cross-resistance, or pleiotropic effect, to seemingly unrelated herbicide chemistries (Beckie and Tardif 2012; Dyer 2018). However, it is not entirely congruous, as one of the two associated resistance traits in this case (meso-R) requires additional genes to confer whole-plant resistance.

A similar association of herbicide-resistance traits was previously reported in *A. tuberculatus* (Tranel et al. 2017), in which a possible genetic association between target site–mediated resistances to ALS- and PPO-inhibiting herbicides was investigated. However, unlike our current study, the genetic association of these resistance traits was attributed to an actual physical linkage of the two genes (195 kb apart) based on analysis of the cultivated grain amaranth (*Amaranthus hypochondriacus* L.) genome sequence (Tranel et al. 2017). Because the precise number and identity of meso-R genes in *Amaranthus* is currently

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unknown and an *A. tuberculatus* draft genome is not yet publicly available, research in the near future aimed at investigating potential physical linkages between metabolic atz-R and meso-R genes in *A. tuberculatus* or *A. hypochondriacus* must be based on probabilistic measures (Ott and Longnecker 2010), as opposed to molecular marker information. By contrast, a single P450 gene (*Nsf1*) controls tolerance to mesotrione, tembotrione, and several other POST herbicides in corn (Nordby et al. 2008; Williams and Pataky 2010).

Summary and Management Implications

Our findings shed new light on the observations from several field populations of dioecious *Amaranthus* species in which mesotrione resistance has yet to be uncoupled from metabolic atz-R (Heap 2020). Our genetic model postulates that qualitative atz-R is an essential component of the more quantitative nature of meso-R observed in *A. tuberculatus*, similar to proposed pathogen-resistance models in crops (Nelson et al. 2018). Future research in our lab will be directed toward utilizing the *AtuGSTF2* gene as an expressed marker to investigate whether meso-R plants selected from our existing F_2 population exhibit elevated *AtuGSTF2* transcript levels, as reported in our previous work with metabolic atz-R plants (Evans et al. 2017). Ultimately, to comprehensively understand the complex molecular–genetic mechanisms underlying these two associated metabolic resistance traits, additional crosses should be performed to generate more F_1 and F_2 populations from the MCR field population as well as from other HPPD inhibitor–resistant and metabolic atz-R *Amaranthus* populations. Following identification of the actual meso-R genes and release of a draft genome sequence, the chromosomal locations of the genetic loci containing these genes and the atz-R locus can be investigated within the *A. tuberculatus* genome.

The inability to uncouple these two metabolic resistance traits in the MCR-derived F_2 population underscores the importance of incorporating integrated weed management systems that use herbicides with different biokinetic properties (i.e., diverse herbicide sites of action and detoxification pathways) in conjunction with nonchemical control measures. Many popular corn PRE herbicide mixtures contain atrazine and mesotrione (as well as other HPPD-inhibiting herbicides), so it is critical to further understand the association between mesotrione and atrazine metabolic resistances to aid in stewardship and reduce selection pressure for additional multiple-resistant *A. tuberculatus* populations. Although the synergistic interaction between atrazine and HPPD-inhibiting herbicides is frequently utilized for weed management in corn production systems (Abendroth et al. 2006; Woodyard et al. 2009), different PSII inhibitors such as metribuzin (O'Brien et al. 2018) or bromoxynil could also be used to reduce the selection for metabolic atrazine resistance in *Amaranthus*.

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