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Glyphosate Resistance in Tall Waterhemp (*Amaranthus tuberculatus*) from Mississippi is due to both Altered Target-Site and Nontarget-Site Mechanisms

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A tall waterhemp population from Missisippi was suspected to be resistant to glyphosate. Glyphosate dose response experiments resulted in GR_{50} (dose required to reduce plant growth by 50%) values of 1.28 and 0.28 kg ae ha^{-1} glyphosate for the glyphosate-resistant (GR) and -susceptible (GS) populations, respectively, indicating a five-fold resistance. The absorption pattern of ^{14}C-glyphosate between the GR and GS populations was similar up to 24 h after treatment (HAT). Thereafter, the susceptible population absorbed more glyphosate (55 and 49% of applied) compared to the resistant population (41 and 40% of applied) by 48 and 72 HAT, respectively. Treatment of a single leaf in individual plants with glyphosate at 0.84 kg ha^{-1}, in the form of 10 1-µl droplets, provided greater control (85 vs. 29%) and shoot fresh weight reduction (73 vs. 34% of nontreated control) of the GS plants compared to the GR plants, possibly indicating a reduced movement of glyphosate in the GR plants. The amount of ^{14}C-glyphosate that translocated out of the treated leaves of GR plants (20% of absorbed at 24 HAT and 23% of absorbed at 48 HAT) was significantly lower than the GS plants (31% of absorbed at 24 HAT and 32% of absorbed at 48 HAT). A potential difference in shikimate accumulation between GR and GS populations at different concentrations of glyphosate was also studied in vitro. The IC_{50} (glyphosate concentration required to cause shikimate accumulation at 50% of peak levels measured) values for the GR and GS populations were 480 and 140 µM of glyphosate, respectively, resulting in more shikimate accumulation in the GS than the GR population. Sequence analysis of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), the target site of glyphosate, from GR and GS plants identified a consistent single nucleotide polymorphism (T/C, thymine/cytosine) between GR/GS plants, resulting in a proline to serine amino acid substitution at position 106 in the GR population. The GR and GS plants contained equal genomic copy number of *EPSPS*, which was positively correlated with *EPSPS* gene expression. Thus, glyphosate resistance in the tall waterhemp population from Mississippi is due to both altered target site and nontarget site mechanisms. This is the first report of an altered *EPSPS*-based resistance in a dicot weed species that has evolved resistance to glyphosate.

Nomenclature: Glyphosate; tall waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer.

Key words: Absorption, autoradiography, EPSPS, gene amplification, mutation, phosphorimaging, shikimate, translocation.

Glyphosate, a nonselective, broad-spectrum, systemic, POST herbicide. has been used extensively throughout the world in both crop and noncrop lands since its commercialization in 1974. With the introduction of glyphosate-resistant (GR) crops in the mid-1990s, glyphosate has been used selectively and predominantly for weed control in GR crops without concern for crop injury. The widespread adoption of GR crops around the world has resulted in the evolution of several GR weed biotypes.

As of August 2012, 24 weed species are reported to be resistant to glyphosate worldwide (Heap 2012). Among these weeds, GR Palmer amaranth (*Amaranthus palmeri* S. Wats.) has gained notoriety due to its aggressive growth habit and fecundity, economic impact on row crop production systems of southeastern United States, propensity to develop multiple herbicide resistance, and ability to hybridize with other pigweeds belonging to the *Amaranthus* genus. For example, hybridization of Palmer amaranth has been reported with smooth pigweed (*Amaranthus hybridus* L.) (Gaines et al. 2011), spiny amaranth (*Amaranthus spinosus* L.) (Gaines et al. 2011), and tall waterhemp (Franssen et al. 2001; Wetzel et al. 1999). Another pigweed, tall waterhemp, has growth characteristics similar to Palmer amaranth. Tall waterhemp has been shown to hybridize with smooth pigweed (Trucco et al. 2005a,b, 2009) in addition to Palmer amaranth. Populations of tall waterhemp have developed resistance to glyphosate in several states in the United States, including Illinois, Indiana, Iowa, Kansas, Minnesota, Missouri, North Dakota, Oklahoma, South Dakota, Tennessee, and Texas (Heap 2012; Legleiter and Bradley 2008; Light et al. 2011). In the summer of 2008, seed samples of a tall waterhemp population suspected to be resistant to glyphosate were collected from a GR soybean field in southern Washington County, Mississippi.

In this article we report characterization of glyphosate resistance in the above tall waterhemp population. The objectives of this research were (1) to determine if glyphosate resistance exists in a tall waterhemp population from Mississippi and to quantify the level of resistance; and (2) to elucidate the physiological and molecular mechanism of glyphosate resistance in the tall waterhemp population.

Materials and Methods

Seed Collection, Storage, Germination, Planting, Growth, and Herbicide Treatment Conditions. In the summer of 2008, seed from tall waterhemp plants suspected to be resistant to glyphosate was randomly collected from a field that had been continuously planted to GR soybean in 2008 and several years prior. Tall waterhemp inflorescence spikes containing seeds were air-dried in a greenhouse (25/20 C day/night, 12-h photoperiod under natural sunlight conditions) for 7 d, cleaned, and stored at 2 to 8 C until further use. Germination of seeds, transplanting of seedlings, growth of

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plants, and all experiments were conducted under these growing conditions unless otherwise described.

For ^{14}C-glyphosate absorption and translocation experiments, plants were transferred from the greenhouse to a growth chamber 2 d prior to ^{14}C-glyphosate application for acclimatization. The growth chamber was maintained at 25/20 C with a 13-h photoperiod (600 µmol m^{-2} s^{-1}) provided by fluorescent and incandescent bulbs. Plants were left in the growth chamber until harvest. Seeds were planted at 1-cm depth in 50 cm by 20 cm by 6 cm plastic trays with holes containing a commercial potting mix (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA 98008). Two wk after emergence, tall waterhemp plants were transplanted into 6 cm by 6 cm by 6 cm pots containing the potting mix mentioned before. Plants were fertilized once with a nutrient solution (Miracle-Gro, The Scotts Company LLC, Marysville, OH 43041) containing 200 mg L^{-1} each of N, P_2O_5, and K_2O 1 wk after transplanting and subirrigated as needed thereafter. All herbicide treatments were applied with a moving nozzle sprayer equipped with 8002E nozzles (Spraying Systems Co., Wheaton, IL 60189) delivering 140 L ha^{-1} at 280 kPa to tall waterhemp plants that were 10 cm tall and at the four- to six-leaf stage. Either percent control (visible estimate of injury on a scale of 0 [no injury] to 100 [plant death]) or aboveground shoot fresh weight reduction (expressed in terms of nontreated control plants), or both, was recorded 3 wk after treatment (WAT). A known glyphosate-susceptible (GS) tall waterhemp population was obtained from Missouri (K. Bradley, University of Missouri) and was included for comparison in all experiments. At the time of initiating this research, information was lacking on reliable wild type/susceptible populations from Mississippi. All studies were conducted during the months of January to April and August to November in 2009 to 2012.

Screening of Populations with a Discriminating Glyphosate Dose. In preliminary resistance screening studies, several tall waterhemp plants were treated with a 0.84 kg ae ha^{-1} rate of glyphosate (potassium salt, Roundup WeatherMAX®, Monsanto Company, St. Louis, MO 63167) (data not shown). Plants that survived 3 WAT were allowed to grow and randomly cross with each other to produce the second generation seed. Additional screening experiments indicated that all the second generation plants survived a glyphosate treatment of 0.84 kg ha^{-1} (data not shown). This second generation seed was used in all subsequent studies.

Glyphosate Dose Response. GR and GS tall waterhemp plants were treated with glyphosate at 0, 0.21, 0.42, 0.84, 1.68, and 3.36 kg ha^{-1}. Percent control ratings were recorded 3 WAT. The 0.84 kg ha^{-1} rate represents the normal (1×) field application rate. There were three replications per treatment and the experiment was conducted three times.

^{14}C-Glyphosate Absorption and Translocation. GR and GS tall waterhemp plants were treated with glyphosate as described before, except that the third fully expanded leaf was covered with a clear plastic sleeve. This sleeve was removed immediately after herbicide treatment for subsequent (within 30 min of overspray) application of solutions containing ^{14}C-glyphosate (^{14}C-methyl labeled with 2.0 GBq $mmol^{-1}$ specific activity, 99.5% radiochemical purity in an aqueous stock solution of 7.4 MBq ml^{-1}, American Radiolabeled Chemicals, Inc., St. Louis, MO 63146). A solution containing glyphosate at a final concentration equivalent to 0.84 kg ha^{-1} in 140 L was prepared using ^{14}C-glyphosate, a commercial formulation of glyphosate, and distilled water. A 10-µl volume of the treatment solution, containing 5 kBq of ^{14}C-glyphosate, was applied to the adaxial surface of the third true leaf of 10-cm-tall plants in the form of 25 droplets with a micro applicator. Plants were harvested at 1, 4, 8, 24, 48, and 72 h after ^{14}C-glyphosate treatment (HAT) for absorption measurement. ^{14}C-glyphosate-treated plants, harvested 24 and 48 HAT, were divided into treated leaf, shoot above treated leaf (SATL), shoot below treated leaf (SBTL), and roots for measuring translocation. The treated leaf was immersed in 10 ml 10% methanol in a glass vial and gently shaken for 20 s to remove nonabsorbed ^{14}C-glyphosate remaining on the leaf surface. The washed leaf was rewashed with an additional 10 ml of 10% methanol. Two 1-ml aliquots of each leaf wash were mixed with 10 ml scintillation cocktail (Ecolume, ICN, Costa Mesa, CA 92626). The plant parts were wrapped in a single layer of tissue paper (Kimwipes, Kimberly-Clark Corporation, Roswell, GA), placed in a glass vial, and oven dried at 60 C for 48 h. Oven-dried plant samples were combusted in a biological oxidizer (Packard Instruments Company, Downers Grove, IL) and the evolved $^{14}CO_2$ was trapped in 10 ml Carbosorb E (Packard BioScience Company, Meridian, CT 06450) and 10 ml Permaflour E+ (Packard BioScience). Radioactivity from leaf washes and oxidations was quantified using liquid scintillation spectrometry. The average recovery of applied ^{14}C-glyphosate was 95%, based on the sum of the radioactivity measured in all plant parts (absorption, expressed as percent of applied ^{14}C) and leaf washes. Total of radioactivity recovered in all plant parts except the treated leaf was designated as translocated ^{14}C and expressed as percent of absorbed. There were four to seven replications per treatment and the experiment was conducted twice.

Phosphorimaging. GR and GS tall waterhemp plants were treated with a solution containing ^{14}C-glyphosate as described before. The treated leaves from the plants were removed at 24 and 48 HAT to wash off unabsorbed radioactivity. Roots were gently rinsed with water to remove soil and blotted dry with paper towels. Plants were then mounted on a 27 by 21.25 cm piece of plain white paper and shoot and root parts were evenly spread and kept in place with thin strips of clear office tape. Care was taken to avoid contact of the washed treated leaf with other parts of the plant. The mounted plant was pressed between one or more layers of newspaper, and bookended with two hard cardboard sections. The assembled plant press was held together with large binder clips and stored at −20 C for later drying. The plant samples were dried in a gravity convection oven set at 60 C for 24 h. Phosphorimaging was used to develop an image of the plant samples. After cooling the dried sample to room temperature, the plant was placed in a 20 by 40 cm exposure cassette (GE Healthcare Bio-Sciences Corp., Piscataway, NJ 08855) and brought into contact with a storage phosphor screen (BAS IP SR 2025 E, GE Healthcare Bio-Sciences Corp.) under diffused lighting. The apparatus was placed in a dark cabinet for 24 h. A phosphosimager (Typhoon FLA 7000, GE Healthcare) was used to detect distribution of ^{14}C-glyphosate and develop an image. There were two replications per timing

for each of GR and GS populations and the experiment was conducted twice.

Efficacy of Single Leaf-Treated Glyphosate on Whole Plant. GR and GS tall waterhemp plants were treated with a glyphosate solution at a concentration equivalent to 0.84 kg ha^{-1} (1× the normal field rate) in 140 L of water. Ten µl of the glyphosate solution was placed on the adaxial surface of a third fully expanded leaf as 10 droplets. At 3 WAT, percent control was visually estimated and the aboveground shoot was clipped and fresh weight was recorded (to calculate shoot fresh weight reduction). There were three replications per treatment and the experiment was conducted twice.

Shikimate Assay with Leaf Discs. Shikimate assay on tall waterhemp populations was conducted following previously reported protocols (Shaner et al. 2005). Leaf discs (6-mm-diam) were excised from leaves with a common hand-held single-hole paper punch. Twenty leaf discs were added to 20 mL glass vials containing 1 ml of 10 mM ammonium phosphate (pH 4.4) plus 0.1% (v/v) Tween 80 surfactant solution and various concentrations of glyphosate (0, 7.8, 31.2, 125, 500, and 1,000 µM). Vials were then placed in a controlled environment chamber equipped with fluorescent and incandescent bulbs (400 µmol m^{-2} s^{-1}) for 16 h at 25 C. Immediately after the 16-h incubation period, vials were frozen. Soon thereafter, 250 µl of 1.25 N HCl was added to each vial and vials were thawed at 60 C for 15 min. The leaf discs turned gray, indicating complete penetration of leaf by the acid. Shikimate was then determined spectrophotometrically (Synergy HT Microplate Reader, BioTek Instruments, Inc., Winooski, VT 05404) following the procedure of Cromartie and Polge (2000). Vials were vortexed and a 25-µl aliquot from the vial was added to an individual well of a 96-well microtiter plate containing 100 ml of a mixture of 0.25% (w/v) periodic acid (Sigma Chemical Co., St. Louis, MO 63103) and 0.25% (w/v) sodium *m*-periodate (Sigma Chemical Co.). The microtiter plate was incubated at room temperature (25 C) for 90 min and was followed by the addition of 100 µl of a mixture of 0.6 N sodium hydroxide and 0.22 M sodium sulfite. The optical density (OD) of the solutions in the microtiter plate wells was measured at 380 nm within 30 min. Background OD was subtracted from readings of the glyphosate treatments. There were four replications per treatment and the experiment was conducted twice.

***EPSPS* Sequence Analysis.** The sequences of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene of two Palmer amaranth sequences (FJ861242.1 and FJ861243.1) and two tall waterhemp mRNA sequences (FJ869880.1 and FJ869881.1) were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and used to generate a single consensus sequence. This consensus sequence was used to design primers to amplify sections of the *EPSPS* mRNA from GR and GS tall waterhemp plants. The four reference sequences ranged from 1,599 base pairs (bp) to 1,967 bp in length. The two Palmer amaranth sequences were longer (approximately 140 bp longer on the 5′ end and approximately 220 bp longer on the 3′ end). The Palmer amaranth sequences also included the 3′ poly-A tail. Overall, the four sequences were highly similar. Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). DNA and RNA were isolated from individual GR and GS tall waterhemp plants using a Maxwell 16™ automated nucleic acid isolation machine (Promega, Madison, WI) following manufacturer's protocols. cDNA synthesis was accomplished using the *EPSPS*-specific primers and 1-step reverse transcriptase-polymerase chain reaction (RT-PCR) following manufacturer's protocols (Thermo Scientific Verso RT-PCR system, Thermo Fisher Scientific Inc., Pittsburgh, PA). All polymerase train reaction (PCR) amplifications were performed on a MJ Research PTC 225 (Biorad, Hercules, CA). Additional genomic DNA fragments were amplified using a hot-start, high fidelity polymerase (JumpStart™ REDTaq® DNA Polymerase, Sigma-Aldrich, St. Louis, MO) using conditions of 95 C for 120 s; 35 cycles of 94 C for 30 s, 57 C for 30 s, 72 C for 120 s; and one cycle of 72 C for 300 s followed by maintenance at 4 C. All amplicons were purified on 1.5% agarose gels using an equal mixture of low melting agarose (BP-1360, Thermo Fisher Scientific Inc.) and multipurpose agarose (BP-160, Thermo Fisher Scientific Inc.). Bands were cut out of the gels and purified using a QIAquick Gel Extraction Kit (#28706, Qiagen, Valencia, CA) following kit protocols. Purified amplicons were cloned using a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) following manufacturer's protocols. For each amplicon, at least three colonies were picked and individually cultured. Plasmid DNA was isolated using a modified alkaline-lysis method and then the forward and reverse sequence of each cloned amplicon was obtained in an ABI 3730xl sequencer (Life Technologies, Carlsbad, CA) using a BigDye v3.1 Terminator/Buffer Ready Rxn Cycle Sequencing kit (Life Technologies) by the Genomics and Bioinformatics Research Unit, Stoneville MS. Sequences were analyzed using Sequencher software (Ver. 5.0, Ann Arbor, MI). Although the entire tall waterhemp *EPSPS* cDNA was cloned and sequenced from a selection of GR and GS plants, the only consistent sequence difference was found in a 192 bp fragment generated by the primers 5′-TTGGACGCTCTCAGAACTCTTGGT-3′ and 5′-TGAATTTCCTCCAGCAACGGCAAC-3′. This specific amplicon was cloned, sequenced, and analyzed from a total of 31 GS plants and 23 GR plants.

***EPSPS* Copy Number and Expression.** Standard DNA and RNA extraction and quantification procedures were employed. Briefly, leaf tissue sample were immediately frozen and ground in liquid nitrogen, genomic DNA was extracted using the DNEasy Plant Mini Kit (Qiagen, Valencia, CA 91355), and total RNA was isolated by the Trizol method (Invitrogen, Carlsbad, CA, 92008) according to the manufacturer's instructions. The RNA samples were treated with the RNase-Free DNase (Qiagen) and then purified using the RNeasy Plant Mini Kit (Qiagen). The quality and quantity of prepared genomic DNA and total RNA were accessed according to the MIQE Guidelines (Bustin et al. 2009). The DNA and RNA were quantified using a NanoDrop (ND-1000) spectrophotometer (Thermo Scientific, Wilmington, DE 19810) and checked for quality and integrity by gel electrophoresis. Quantitative real-time PCR (qPCR) was used to measure *EPSPS* genomic copy number relative to *ALS* (acetolactate synthase, monogenic) and gene expression level of *EPSPS* relative to *ALS* in GR and GS tall waterhemp plants according to previously described procedures (Gaines et al. 2010) using primer sets EPSF (5′-ATGTTGGACGCTCT-

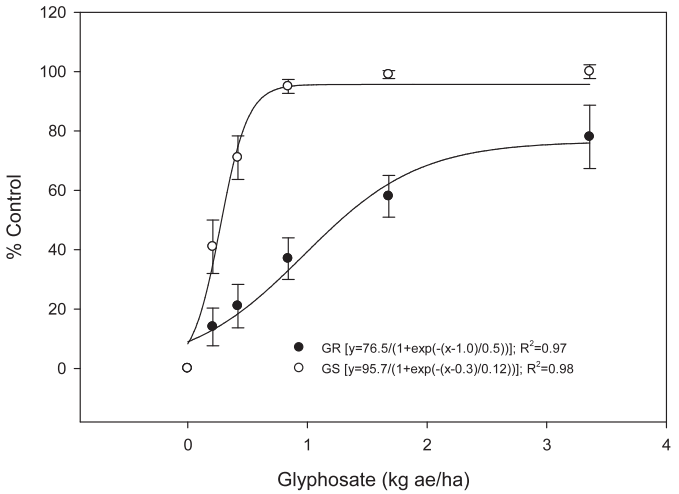

Figure 1. Glyphosate dose response on control of glyphosate-resistant (GR) and -susceptible (GS) tall waterhemp populations 3 wk after treatment. GR_{50} (dose required to reduce plant growth by 50%) values for GR and GS biotypes were 1.28 and 0.28 kg ae ha^{-1} glyphosate, respectively. Vertical bars represent standard error of mean.

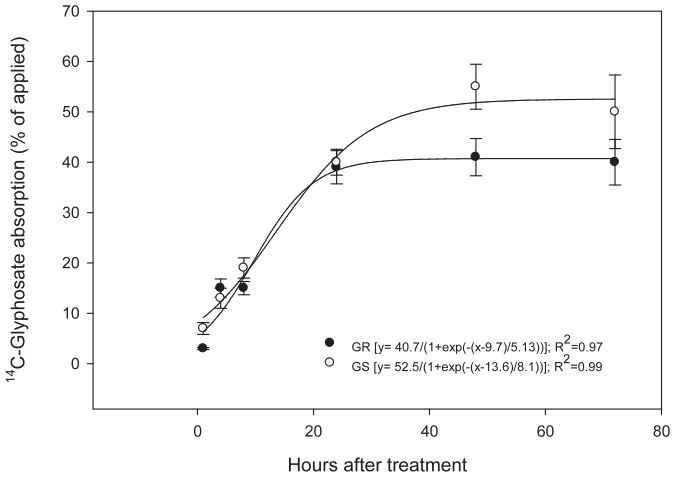

Figure 2. Absorption pattern of ^{14}C-glyphosate in glyphosate-resistant (GR) and -susceptible (GS) tall waterhemp populations. Vertical bars represent standard error of mean.

CAGAACTCTTGGT-3′) × EPSR (5′-TGAATTTCCTC-CAGCAACGGCAA-3′) (195-bp product) and ALSF (5′-GCTGCTGAAGGCTACGCTCG-3′) × ALSR (5′-GCG-GGACTGAGTCAAGAAGTG-3′) (118-bp product) established by Gaines et al. (2010). To measure *EPSPS* genomic copy number, primer efficiency curves were first conducted using a five-fold serial dilution of genomic DNA samples, ranging from 0.08 ng to 50 ng. Primer efficiency and slope were 102.2% and −3.271 (R^2 = 0.996) for *EPSPS* and were 107.7% and −3.150 for ALS (R^2 = 0.990). The qPCR was performed in a 25-µl reaction containing 10 ng genomic DNA and Bio-Rad iQ SYBR Green Supermix. Real-time PCR detection was performed in a Bio-Rad MiniOpticon System PCR machine under the following conditions: 10 min at 94 C, 40 cycles of 94 C for 15 s and 60 C for 1 min then increasing the temperature by 0.5 C every 5 s to access the product melt-curve. A negative control reaction in the absence of template (no template control) was also routinely performed in triplicate for each primer pair resulting in no amplification products. Data was analyzed using CFX manager software (version 1.5). Relative quantification of *EPSPS* was calculated as Δ^{Ct} = (Ct, *EPSPS* − Ct, *ALS*) according to the method described by Gaines et al. (2010). Results were expressed as fold changes in *EPSPS* copy number relative to *ALS*. There were seven replications per population and the experiment was conducted twice. To measure gene expression level, the first strand cDNA was synthesized from 0.5 µg of total RNA in a 20-µl reaction volume using the iScript cDNA synthesis kit (Bio-Rad) per the manufacturer's instructions. The reaction was then diluted with 230 µl distilled water. A 5 µl of aliquot of the diluted cDNA mixture was used in a 25-µl reaction (equivalent of 10 ng total RNA) for qPCR as described above. Each sample was run in three replicates to calculate the mean and standard error of the change in *EPSPS* copy number and expression.

Statistical Analysis. All experiments were conducted using a completely randomized design. Data from all experiments, with the exception of *EPSPS* sequence analysis, copy number, and expression, were analyzed by ANOVA via the PROC GLM statement using SAS software (version 9.2, SAS Institute, Inc., Cary, NC 27513). No significant experiment effect was observed in repeated experiments; therefore, data from experiments were pooled. Nonlinear regression analysis was applied to fit a sigmoidal log-logistic curve of the form

$$y = a/(1 + \exp[-(x - x_0)/b]) \quad [1]$$

where, a is an asymptote, x and x_0 are the upper and lower response limits with the latter approaching 0, and b is the slope of the curve around x_0, to relate effect of glyphosate dose on tall waterhemp control, HAT on ^{14}C-glyphosate absorption, and glyphosate concentration on shikimate accumulation. Equation parameters were computed using SigmaPlot (version 11.0, Systat Software, Inc., San Jose, CA 95110). Treatment means in selected experiments were separated using Fisher's Protected LSD test at P = 0.05.

Results and Discussion

Glyphosate Dose Response. GR_{50} values for the GR and GS tall waterhemp populations, based on percent control, were 1.28 and 0.28 kg ha^{-1} glyphosate (Figure 1). This indicated that the GR population was five-fold more resistant to glyphosate compared to the GS population. This level of glyphosate resistance is more than that reported for a GR common waterhemp from Texas (Light et al. 2011) that was 2.5-fold more resistant compared to a susceptible counterpart. However, the resistance level is lower than the 9.2- to 19.2-fold resistance level reported in GR common waterhemp populations from Missouri (Legleiter and Bradley 2008). It should be noted that tall and common waterhemp are synonymous according to Bryson and DeFelice (2009). The GR plants survived glyphosate treatment and continued growth to the reproductive phase at all rates of glyphosate. Also, a stimulation of axillary growing points (profuse lateral branching) was commonly observed in the GR population. The GS biotype did not survive glyphosate at 0.84 kg ha^{-1} or higher rates.

^{14}C-Glyphosate Absorption and Translocation. The absorption pattern of ^{14}C-glyphosate in the two tall waterhemp populations was similar up to 24 HAT reaching 39 and 40% of applied radioactivity in the GR and GS populations, respectively (Figure 2). Thereafter, the GS population

Table 1. ^{14}C-glyphosate translocation and distribution in glyphosate-resistant (GR) and -susceptible (GS) tall waterhemp populations.[a,b]

Population	Harvest time	TL	Translocation[c]	SATL	SBTL	Root
	h	% of absorbed				
GR	24	80	20	5	8	7
GS	24	69	31	3	18	10
LSD (0.05)		6	6	1	3	4
GR	48	77	23	8	12	3
GS	48	68	32	5	17	10
LSD (0.05)		4	4	2	4	2

[a] Abbreviations: GR, glyphosate-resistant; GS, glyphosate-susceptible, TL, treated leaf; SATL, shoot above treated leaf; SBTL, shoot below treated leaf.
[b] Distribution represents partitioning of absorbed ^{14}C-glyphosate among the treated leaf, shoot above treated leaf, shoot below treated leaf, and root).
[c] ^{14}C-glyphosate outside of treated leaf (shoot above treated leaf, shoot below treated leaf, and root) was considered as translocation.

continued to absorb additional glyphosate reaching 55 and 50% of applied ^{14}C-glyphosate at 48 and 72 HAT, respectively, whereas the uptake of glyphosate in the GR population reached a plateau beyond 24 HAT (41 and 40% of applied by 48 and 72 HAT, respectively). The glyphosate absorption levels reported here for the GS population are similar to those reported in a common waterhemp accession from Missouri that recorded 40.3 to 63.2%, 53 to 65%, and 50.9 to 57.85% of that applied at 26 and 50 HAT, respectively, across three glyphosate formulations (Li et al. 2005).

The amount of ^{14}C-glyphosate that translocated out of the treated leaves of the GR plants (20% of absorbed at 24 HAT and 23% at 48 HAT) was significantly lower than the GS plants (31% of absorbed at 24 HAT and 32% of absorbed at 48 HAT), when compared within harvest times (Table 1). Data from the 24 and 48 HAT was separated due to a significant harvest time effect as well as to enable readership to correlate these results with phosphorimaging data (Figure 3). The glyphosate translocation levels reported here for the GS population are lower than those reported in a common waterhemp accession from Missouri that recorded 49 to 50% of absorbed and 54 to 63% of absorbed at 26 and 50 HAT, respectively, across three glyphosate formulations (Li et al. 2005). Distribution of absorbed ^{14}C-glyphosate in the GR and GS populations is summarized in Table 1. The quantity of ^{14}C-glyphosate that remained in the treated leaf was higher in the GR population (80% of absorbed and 77% of absorbed

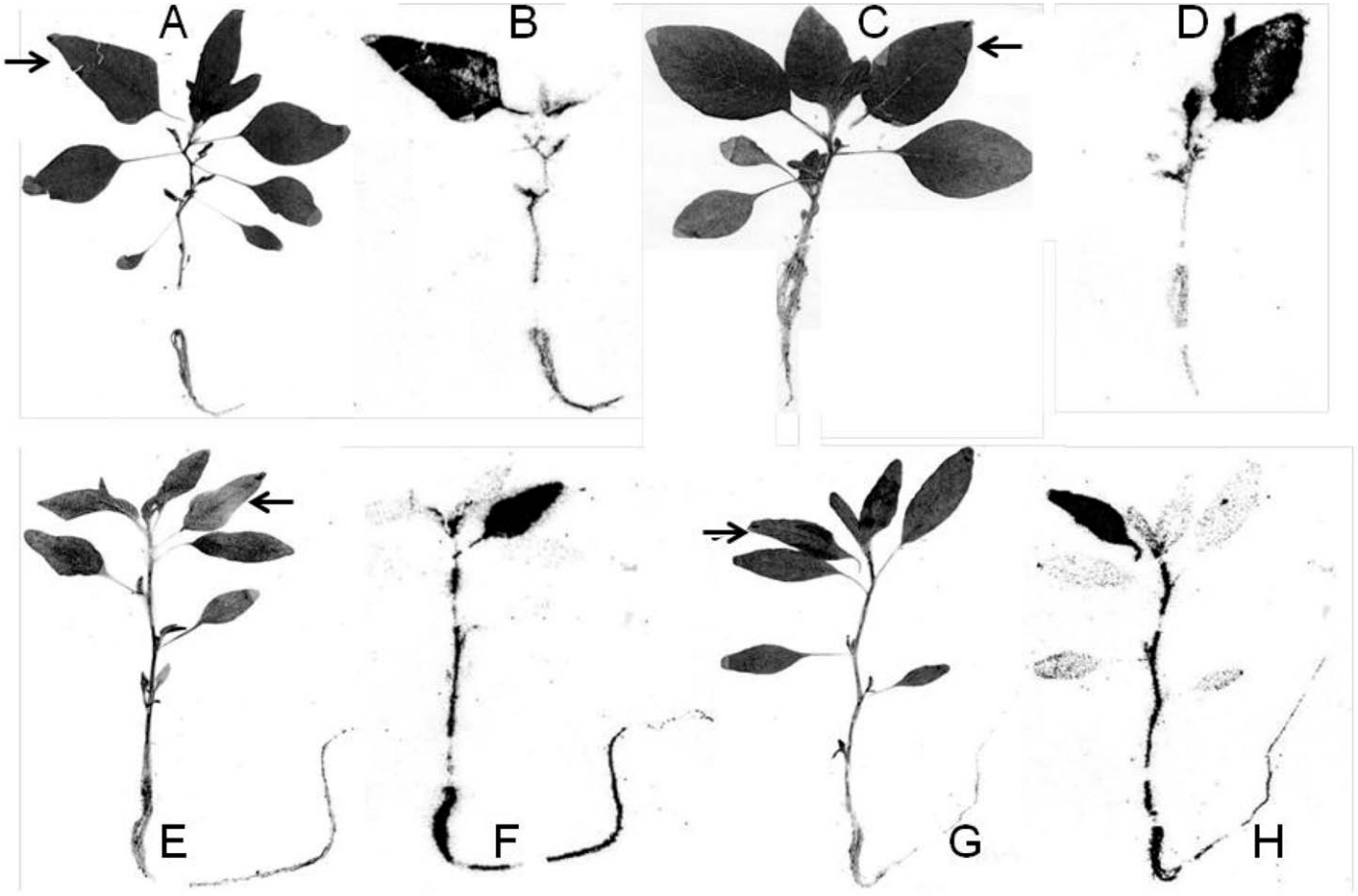

Figure 3. ^{14}C-Glyphosate translocation in: (A to D) glyphosate-resistant (GR), and (E to H) glyphosate-susceptible (GS) tall waterhemp plants. Arrows indicate the treated leaf. First and third columns represent treated plants and second and fourth columns represent their corresponding phosphorimages (autoradiographs) at 24 and 48 h after treatment, respectively.

Table 2. Efficacy of single leaf-treated glyphosate on tall waterhemp populations.[a,b]

Population	Control	Shoot fresh weight reduction
	%	%[c]
GR	29	34
GS	85	73
LSD (0.05)	22	32

[a] Plants (10 cm tall, four to six leaves) were treated with 10 µL of a solution containing a commercial formulation of glyphosate (potassium salt) at a concentration of 0.84 kg ae ha^{-1} in 140 L of water. Ten 1-µl droplets of treatment solution were applied with a microsyringe on the adaxial surface of the third fully expanded leaf.

[b] Abbreviations: GR, glyphosate-resistant; GS, glyphosate-susceptible.

[c] Based on nontreated control plants.

at 24 and 48 HAT, respectively) compared to the GS population (69% of absorbed and 68% of absorbed at 24 and 48 HAT, respectively), within harvest times. Furthermore, the amounts of ^{14}C-glyphosate that accumulated in the shoot above treated leaf and shoot below treated leaf were lower in the GR population compared to the GS population within each tissue and harvest time (24 and 48 HAT). Also, roots of GR plants had less ^{14}C-glyphosate translocated at 48 HAT compared to the GS plants.

Phosphorimaging was used to generate autoradiographs of GR and GS plants treated with ^{14}C-glyphosate, shown in Figure 3, to visualize ^{14}C-glyphosate translocation patterns. ^{14}C-glyphosate mostly remained in the treated leaf of the GR plants with some movement to the meristematic growing points and root at 24 (Figures 3A and 3B) and 48 HAT (Figures 3C and 3D). An interesting feature is that ^{14}C-glyphosate accumulation seems to have shifted from the stem and roots to the primary growing point from 24 to 48 HAT in the GR plants. Thus, despite accumulation of glyphosate in the primary growing point, GR plants could have been able to survive by stimulation of lateral axillary meristems. The GS plants, on the other hand, exhibited discernible movement of ^{14}C-glyphosate in to the stem and leaves more so than the GR plants. Within the GS population, glyphosate moved into other mature leaves by 48 HAT (Figures 3G and 3H) compared at 24 HAT (Figures 3 E and 3F).

The observed difference in the absorption of ^{14}C-glyphosate of GR and GS tall waterhemp populations could be due to variation in the qualitative and quantitative composition of the epicuticular wax, which warrants further investigation, as well as due to a source–sink concentration gradient being steeper in the GS than the GR population. A self-limiting systemic herbicide such as glyphosate is subject to feedback inhibition. In other words, if the concentration of glyphosate in the sink tissues reaches saturating levels, its unloading out of the source (treated) leaf is restricted under certain conditions. There was a clear difference in the translocation of glyphosate between the GR and GS plants, with more remaining in the treated leaf and a smaller amount moving throughout the rest of the GR plants. Similar findings were reported for glyphosate-resistant rigid ryegrass (*Lolium rigidum* Gaudin) (Lorraine-Colwill et al. 2003; Wakelin et al. 2004), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] (Nandula et al. 2008; Perez-Jones et al. 2007), horseweed [*Conyza canadensis* (L.) Cronq.] (Dinelli et al. 2006; Feng et al. 2004; Koger and Reddy 2005), and hairy fleabane [*Conyza bonariensis* (L.) Cronq.] (Dinelli et al. 2008),

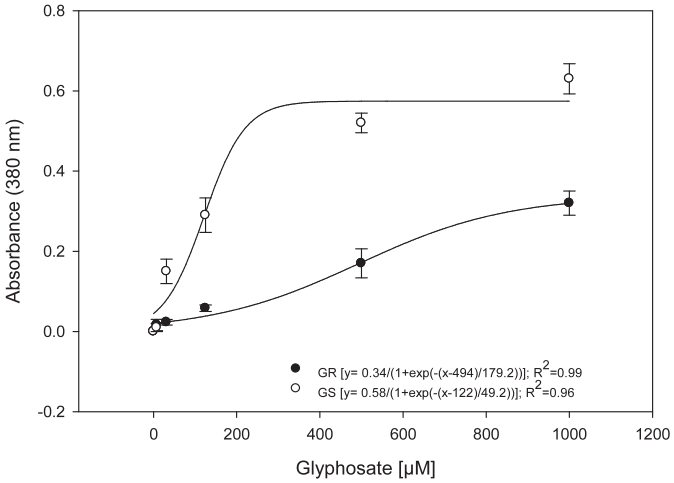

Figure 4. Effect of glyphosate concentration on shikimate levels in excised leaf discs of glyphosate-resistant (GR) and -susceptible (GS) tall waterhemp populations. Vertical bars represent standard error of mean.

where the resistant accessions accumulated less glyphosate compared to their respective susceptible equivalents.

Efficacy of Single Leaf-Treated Glyphosate on the Whole Plant. Treating a single leaf with the normal (1×) glyphosate rate, 0.84 kg ha^{-1}, as 10 1-µl droplets, resulted in 85% control and 73% (of nontreated control plants) reduction in shoot fresh weight of the GS tall waterhemp plants 3 WAT. Conversely, the GR plants were controlled only 29% and had 34% reduction in shoot fresh weight, both significantly less than the GS plants (Table 2). These observations provide indirect evidence of reduced movement of glyphosate from the treated leaf to other plant parts in the GR compared to the GS population. Similar results were reported for horseweed (Koger and Reddy 2005), Italian ryegrass (Nandula et al. 2008), and Palmer amaranth (Nandula et al. 2012), where susceptible biotypes were severely injured (> 80%) or completely controlled (100%) and resistant biotypes of horseweed (Koger and Reddy 2005), Italian ryegrass (Nandula et al. 2008), and Palmer amaranth (Nandula et al. 2012) were controlled/injured only 38 to 58%, 35 to 55%, and 0 to 18%, respectively.

Shikimate Assay with Leaf Discs. Shikimate (shikimic acid) accumulation pattern in the GR and GS tall waterhemp populations is depicted in Figure 4. The IC_{50} (glyphosate concentration required to cause shikimate accumulation at 50% of peak levels measured) values for the GR and GS populations were 480 and 140 µM of glyphosate, respectively. It was apparent that the GR population accumulated lower levels of shikimate compared to the GS population, confirming the former's lesser sensitivity to glyphosate.

***EPSPS* Sequence Analysis.** Primers were designed to amplify mRNA fragments covering approximately 1,300 bp beginning approximately 100 bp in from the 5′ end of the composite mRNA reference sequence. Fragments were amplified, cloned, and sequenced from ten known GS plants and seven known GR plants. When aligned, the sequences were very similar; however, there was one nucleotide that was consistently different between individual GS and GR plants in a 192 bp

Table 3. (A) Nucleotide sequence and (B) translation of selected reference fragments and the consensus sequences of glyphosate-resistant (GR) and -susceptible (GS) tall waterhemp plants for the same region of the 5-enolpyruvylshikimate-3-phosphate synthase gene.[a]

Consensus reference nucleotide sequence[b]	C T T	G G A	A A T	G C A	G G A	A C A	G C G	A T G	C G C	C C A	T T G	A C A	G C T	G C G	G T T
A. Nucleotide sequence															
Amaranthus tuberculatus biotype ACR (susceptible): FJ869880.1	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —
A. tuberculatus biotype WCS (susceptible): FJ869881.1	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —
A. palmeri (susceptible): FJ861242.1	— — —	— —T	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —
A. palmeri (resistant): FJ861243.1	— — —	— — T	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —
Eleusine indca (susceptible)[c]	T — G	— — G	— — —	— — T	— — —	— — T	— — A	— — —	— — A	— — —	— — —	— — —	— — A	— — C	— — A
E. indica (resistant)[c]	T — G	— — G	— — —	— — T	— — —	— — T	— — A	— — —	— — A	T — —	— — —	— — —	— — A	— — C	— — A
Lolium rigidum (susceptible)[d]	T — G	— — G	— — C	— — T	— — —	— — T	— — —	— — —	— — G	— — —	— — —	— — G	— — A	— — T	— — A
L. rigidum (resistant)[d]	T — G	— — G	— — C	— — T	— — —	— — T	— — —	— — —	— — G	T — —	— — —	— — G	— — A	— — A	— — A
A. tuberculatus (GS consensus)[e]	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —
A. tuberculatus (GR consensus)[e]	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	T — —	— — —	— — —	— — —	— — —	— — —
Amino acid location	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
B. Translation[f,g]															
Arabidopsis thaliana: NM_130093.2	L	G	N	A	G	T	A	M	R	P	L	T	A	A	V
Amaranthus tuberculatus biotype ACR (susceptible): FJ869880.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. tuberculatus biotype WCS (susceptible): FJ869881.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. palmeri (susceptible): FJ861242.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. palmeri (resistant): FJ861243.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Eleusine indca (susceptible)[c]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
E. indica (resistant)[c]	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—
Lolium rigidum (susceptible)[d]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L. rigidum (resistant)[d]	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—
A. tuberculatus (GS consensus)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. tuberculatus (GR consensus)	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—

[a] Abbreviations for A. Nucleotide sequence: A. Adenine; C, Cytosine, G, Guanine; T, Thymine.
[b] The consensus sequence of the highly conserved region of the *EPSPS* gene from two *Amaranthus tuberculatus* and two *A. palmeri* sequences.
[c] The susceptible (S) and resistant (R) sequences of *Eleusine indica* reported by Baerson et al. (2002).
[d] The susceptible (S) and resistant (R) sequences of *Lolium rigidum* reported by Simarmata and Penner (2008).
[e] The susceptible (GS) and resistant (GR) sequences of *A. tuberculatus* evaluated in this study. All S plants had the cytosine nucleotide and all R plants had the thymine nucleotide.
[f] The protein translations of the nucleotide sequences as aligned with *Arabidopsis thaliana*: NM_130093.2.
[g] Abbreviations for B. Translation: A, alanine; G, glycine; L, leucine; M, methionine; N, asparagine; P, proline; R, arginine; T, threonine; V, valine.

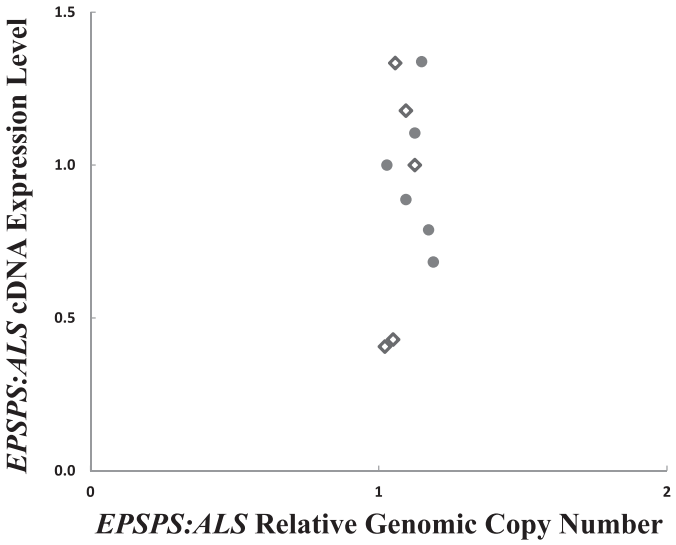

Figure 5. No increase in *EPSPS* relative genomic copy number or increase in *EPSPS* cDNA expression levels occurred in: (GR, filled circles) glyphosate-resistant, and (GS, open diamonds) glyphosate-susceptible tall waterhemp populations.

fragment amplified by the primers 5′-TTGGACGCTCTCA-GAACTCTTGGT-3′ and 5′-TGAATTTCCTCCAGCAA-CGGCAAC-3′. The consistent nucleotide change was from a cytosine (C) in susceptible plants to a thymine (T) in resistant plants. The primers were also found to amplify the same 192 bp fragment in gDNA. The fragment was amplified, cloned, and sequenced from the gDNA of an additional 21 GS plants and 16 GR plants. All 21 GS plants had the same respective nucleotide (cytosine) as in the GS mRNA and all 16 GR plants all had the same respective nucleotide (thymine) as in the GR mRNA. The consensus GS and GR nucleotide sequence of the region flanking the mutation along with the references sequences are shown in Table 3, section A.

Alignment and translation of the nucleotide sequences described above (Table 3, section A) indicated that the cytosine to thymine mutation resulted in an amino acid change from proline to serine corresponding to proline 106 in *Arabidopsis thaliana* (L.) Heynh. (NM_130093.2, Table 3, section B). This region is highly conserved (Gaines et al. 2010). For the two reference Palmer amaranth sequences, including the one from the source resistant to glyphosate, there was no amino acid change at the 106 location. The mechanism of resistance in this Palmer amaranth has been shown to be, rather, due to amplified expression of *EPSPS* (Gaines et al. 2010). However, Baerson et al. (2002) and Simarmata and Penner (2008) reported a similar cytosine to thymine single nucleotide mutation that resulted in a proline to serine substitution in a resistant goosegrass [*Eleusine indica* (L.) Gaertn.] biotype and a resistant rigid ryegrass biotype, respectively (Table 3). In both instances, the mutation was at the same position of the highly conserved region of the *EPSPS* gene corresponding to proline 106 (Table 3, section B) and that was identified in the resistant waterhemp plants evaluated in this study. This is the first report of a mutation at the Pro_{106} location in a dicot weed species resistant to glyphosate. All previous reports of mutations at the Pro_{106} site leading to resistance to glyphosate (including the two reports cited above) have been from grasses. In rigid ryegrass, the proline at position 106 has been replaced by alanine (Yu et al. 2007), serine (Bostamam et al. 2012; Simarmata and Penner 2008), or threonine (Bostamam et al. 2012; Wakelin and Preston 2006a) in resistant accessions. In Italian ryegrass, resistance to glyphosate due to a mutated *EPSPS* has been attributed to substitution of Pro_{106} with serine (Jasieniuk et al. 2008; Perez-Jones et al. 2007) or alanine (Jasieniuk et al. 2008; Perez-Jones et al. 2007). Glyphosate-resistant goosegrass has been reported to contain a point mutation at position 106 leading to a proline to serine (Baerson et al. 2002; Kaundun et al. 2008; Ng et al. 2003, 2004) or threonine (Ng et al. 2003, 2004) change. Populations of junglerice [*Echinochloa colona* (L.) Link] resistant to glyphosate from California (Alarcón-Reverte et al. 2013) and Australia (Thai et al. 2012) have been confirmed to possess a proline to serine substitution at position 106.

EPSPS Copy Number and Expression. Genomic estimation of *EPSPS* gene copy number relative to *ALS* using q-PCR showed that GR contained a single copy of the *EPSPS* gene as well as GS plants (Figure 5). Quantitative PCR on cDNA revealed that the single copy of *EPSPS* gene was equally expressed in GR and GS plants. These results differ from the reports of *EPSPS* gene amplification in tall waterhemp (Chatham et al. 2010), Italian ryegrass (Salas et al. 2012), kochia [*Kochia scoparia* (L.) Schrad.] (Westra et al. 2013), and Palmer amaranth (Gaines et al. 2010; Ribeiro et al. 2011). Chatham et al. (2010) speculated about the involvement of an additional resistance mechanism in GR tall waterhemp.

Therefore, these results indicate that the tall waterhemp population from Washington County, Mississippi is five-fold more resistant to glyphosate, compared to a susceptible population. The GR population exhibited reduced translocation of glyphosate as well as contained a proline to serine substitution at location 106 of a highly conserved region of *EPSPS*. This is the first case of an altered *EPSPS*-based resistance in a dicot weed species that has evolved resistance to glyphosate. Thus, the mechanism of resistance to glyphosate in the GR population has been determined to be based on a combination of altered target site (*EPSPS*) and nontarget site (reduced translocation). Other weed species have been reported to possess multiple mechanisms of resistance to glyphosate in the same population/biotype/accession. For instance, two rigid ryegrass populations from Australia accumulated less glyphosate as well as contained a mutation where Pro_{106} was substituted by serine or threonine (Bostamam et al. 2012). In addition, glyphosate resistance levels of these populations were higher than those of two populations that had a single mechanism of glyphosate resistance, modified target site, or reduced translocation. In a similar fashion, it is reasonable to expect that tall waterhemp populations with multiple mechanisms of glyphosate resistance would have higher levels of resistance compared to populations with a single resistance mechanism.

Interspecific hybridization between weed species belonging to the *Amaranthus* genus has been documented before (see Introduction). Gaines et al. (2011) demonstrated that a previously unknown glyphosate resistance mechanism, amplification of *EPSPS* (Gaines et al. 2010), could be transferred from Palmer amaranth to other *Amaranthus* species including tall waterhemp. This could have a profound impact on weed management, especially herbicide-resistant weed control

programs. A grower or a land manager could face a situation of having to deal with a population of tall waterhemp or Palmer amaranth or a mixture of them along with hybrids that could contain genes encoding for multiple mechanisms of resistance to glyphosate.

The fitness costs of acquiring two or more mechanisms of glyphosate resistance in tall waterhemp are not clear. However, research on rigid ryegrass in Australia indicated noticeable fitness differences where glyphosate-resistant plants from a population produced fewer but larger seeds compared to -susceptible plants from the same population (Pedersen et al. 2007); however, there were no growth differences between the resistant and susceptible populations. In a different study, frequency of resistant rigid ryegrass plants decreased over a period of 2 to 3 yr in the absence of selection pressure from glyphosate (Preston et al. 2009; Wakelin and Preston 2006b). These fitness studies involved the reduced translocation of glyphosate mechanism only, which brought about significant fitness penalty (Preston et al. 2009). Thus far, fitness studies on glyphosate-resistant populations of rigid ryegrass or other weed species, such as tall waterhemp containing an altered target site or a combination of two resistance mechanisms, have not been reported.

The level of resistance endowed in a glyphosate-resistant weed species by a modified target site is generally lower than by reduced translocation (Shaner 2010; Shaner et al. 2012). Presence of these two mechanisms of resistance in the GR population makes it difficult to estimate the relative contribution of each mechanism to the overall resistance to glyphosate. This aspect may be addressed in future studies involving reciprocal crosses of the GR and GS populations and characterizing the phenotype of the progeny after treatment with glyphosate or genetic analysis, or both, using molecular markers. Nevertheless, certain conclusions can be drawn from the shikimate and translocation data. The GR population accumulated less shikimate than the GS population, even at higher glyphosate levels, unlike previous reports (Nandula et al. 2008; Perez-Jones et al. 2005). Thus, the glyphosate translocation model proposed by Shaner (2009) that purports existence of a barrier at the cellular level that prevents glyphosate loading into the phloem does not relate to the GR tall waterhemp population. On the other hand, the shikimate results, as well as the reduced translocation of glyphosate in the GR population, indicate the presence of a mechanism of avoidance or reduced glyphosate accumulation into the mesophyll cells and phloem, respectively. The glyphosate in the GR plants could essentially be loaded into a vacuole via a system akin to the sequestration mechanism described in horseweed (Ge et al. 2010) and ryegrass (Ge et al. 2012). Therefore, the above observations are consistent with proposed models of noncellular glyphosate translocation in GR weeds (Shaner 2009; Shaner et al. 2012). Taken altogether, we hypothesize that the reduced translocation could be contributing more to the resistance mechanism than the altered target site in the GR tall waterhemp population.

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