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Target and Nontarget Resistance Mechanisms Induce Annual Bluegrass (*Poa annua*) Resistance to Atrazine, Amicarbazone, and Diuron

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Annual bluegrass is a weed species in turfgrass environments known for exhibiting resistance to multiple herbicide modes of action, including photosystem II (PSII) inhibitors. To evaluate populations of annual bluegrass for susceptibility to PSII inhibitors of varied chemistries, populations were treated with herbicides from triazolinone, triazine, and substituted urea families: amicarbazone, atrazine, and diuron, respectively. Sequencing of the *psbA* gene confirmed the presence of a Ser_{264} to Gly amino acid substitution within populations that exhibited resistance to both atrazine and amicarbazone. A single biotype, DR3, which lacked any previously reported *psbA* gene point mutation, exhibited resistance to diuron, atrazine, and amicarbazone. DR3 had a significantly lower rate of absorption and translocation of atrazine and had enhanced atrazine metabolism when compared with both the Ser_{264} to Gly resistant mutant and susceptible biotypes. We thus report possible nontarget mechanisms of resistance to PSII-inhibiting herbicides in annual bluegrass.
Nomenclature: Amicarbazone; atrazine; diuron; annual bluegrass, *Poa annua* L.
Key words: Herbicide, herbicide resistance, resistance, target-site mutation, turfgrass.

Poa annua es una especie de maleza en ambientes de céspedes conocida por presentar resistencia a múltiples modos de acción de herbicidas, incluyendo inhibidores del fotosistema II (PSII). Para evaluar la susceptibilidad de poblaciones de *P. annua* a inhibidores PSII de diferentes características químicas, varias poblaciones fueron tratadas con herbicidas de las familias triazolinone, triazine, y urea sustituidas: amicarbazone, atrazine, y diuron, respectivamente. La secuenciación del gen *psbA* confirmó la presencia de una sustitución de amino ácidos de Ser_{264} a Gly en poblaciones que presentaron resistencia a atrazine y amicarbazone. Un solo biotipo, DR3, el cual carecía de cualquier reporte previo de mutaciones puntuales en el gen *psbA*, presentó resistencia a diuron, atrazine, y amicarbazone. DR3 tuvo una tasa de absorción y translocación significativamente menor de atrazine y un mayor metabolismo de atrazine cuando se comparó con biotipos resistentes con la mutación Ser_{264} a Gly y con biotipos susceptibles. De esta forma, reportamos posibles mecanismos de resistencia a herbicidas inhibidores de PSII en *P. annua* que no involucran el sitio activo.

Annual bluegrass is a cool-season grass species treated as a weed in most turfgrass circumstances. It rapidly adapts to environmental constraints and commonly infests golf courses, sports fields, and lawns (Casler and Duncan 2003). In dormant stands of bermudagrass (*Cynodon* spp.), annual bluegrass may be controlled through a variety of herbicide regimes to ensure PRE and POST control; however, annual bluegrass has evolved resistance under continual application of chemicals that act on a single site of action (Burgos et al. 2013; Délye et al. 2013; Matzrafi et al. 2014; McElroy et al. 2013; Perry et al. 2012; Yelverton and Isgrigg 1998).

Photosystem II (PSII) inhibitors are commonly used for control of annual bluegrass (Vargas and Turgeon 2003). PSII inhibitors act by blocking electron transfer to the secondary QB plastoquinone (QB-site) acceptor of the D1 subunit protein, resulting in interruption of electron transfer from the primary plastoquinone site (QA-site) to QB (Powles and Yu 2010). The interrupted electron transfer causes oxidative stress, forming free radicals,

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which cause rapid cellular degradation, reducing adenosine triphosphate and nicotinamide adenine dinucleotide phosphate hydrogen synthesis in the chloroplast and an inability to fix CO_2. PSII inhibitors are classified in several chemical families, including triazinones, triazines, uracils, nitriles, benzothiadiazinones, and substituted ureas (Menne 2005).

Herbicide resistance is caused by either target-site mutations or alterations in plant physiology. (Délye et al. 2013; Devine and Shukla 2000; Matzrafi et al. 2014; Powles and Yu 2010). Target-site mutations involve the modification of a binding site preventing the occurrence of specific herbicide interactions (Devine and Shukla 2000). In contrast, nontarget-site resistance involves a change in a plant's physiological response to herbicides, such as altered uptake, translocation, sequestration, or metabolism (Délye 2013; Yuan et al. 2007). Target-site mutations have been reported to yield resistance to inhibitors of acetyl-coenzyme A carboxylase, acetolactate synthetase (ALS), 5-enolpyruvylshikimate-3-phosphate synthase, microtubule assembly, phytoene desaturase, protoporphyrinogen oxidase, and PSII (Arias et al. 2005; Patzoldt et al. 2006; Powles and Yu 2010). Plants with metabolism-based nontarget-site resistance exhibit changes in at least one of three enzymes: glutathione transferases, the glycosyltransferase, or the cytochrome P450 monooxygenases (Yuan et al. 2007).

Annual bluegrass resistant to PSII-inhibiting herbicides has been previously reported in both peer-reviewed literature and the International Survey of Herbicide-Resistant Weeds (Heap 2015). In 1978, resistance to atrazine, a triazine herbicide, was first reported along a highway in Normandy, France (Darmency and Gasquez 1981). Since then, populations of triazine-resistant mutants of annual bluegrass have been reported in the United States in Alabama in 1980, followed by Oregon, North Carolina, Mississippi, Virginia, and Tennessee (Hanson and Mallory-Smith 2000; Heap 2015; Kelly et al. 1999). In addition to France, annual bluegrass has been reported resistant to PSII inhibitors internationally in Belgium, the Czech Republic, Germany, Japan, the Netherlands, Norway, and the United Kingdom (Chodová et al. 1994; De Prado et al. 1996; Heap 2015; Netland 1996; Putwain 1982).

Sequencing of the *psbA* gene in annual bluegrass has indicated two point mutations—Ser_{264} to Gly and Val_{219} to Ile—responsible for resistance to PSII inhibitors (Darmency and Gasquez 1981; Mengistu et al. 2000; Perry et al. 2012). Ser_{264} to Gly mutation imparts cross-resistance to other PSII-inhibiting herbicides, including amicarbazone, classified in the triazolinone family (Dayan et al. 2009; Hutto et al. 2004; Perry et al. 2012; Yu et al. 2013). Annual bluegrass mutants with a Val_{219} to Ile substitution have also been reported resistant to diuron and metribuzin (Mengistu et al. 2000).

Occurrences of resistance to PSII-inhibiting herbicides have been related to increased levels of herbicide detoxification, and although not reported in annual bluegrass, nontarget-site, metabolism-based resistance of PSII inhibiting herbicides has been reported in velvetleaf (*Abutilon theophrasti* Medik.) (Burnet et al. 1993b; Gronwald 1989, 1997; Yuan et al. 2007). Simazine-resistant biotypes of rigid ryegrass (*Lolium rigidum* Gaudin) metabolize simazine at a higher rate than susceptible biotypes do, while maintaining high levels of absorption and translocation (Burnet et al. 1993b). Cytochrome P450 monooxygenases, a wide family of enzymes essential to plant metabolic processes, can endow resistance to multiple herbicides, including PSII inhibitors simazine and metribuzin in rigid ryegrass (Burnet et al. 1993a; De Prado et al. 1997; Preston 2003; Preston et al. 1996). Because herbicide metabolism, uptake, and translocation affects the overall efficacy of herbicides, nontarget mechanisms can also lead to an increase in resistance (Pan et al. 2012; Matzrafi et al. 2014; Yu et al. 2013).

Because of discovering annual bluegrass populations resistant to other modes of action (Brosnan et al. 2012, 2015; Cutulle et al. 2009; McElroy et al. 2013), there is renewed interest in using PSII-inhibiting herbicides for annual bluegrass control. Further, amicarbazone is a relatively new PSII-inhibiting herbicide, and it has only been evaluated for response to Ser_{264} to Gly mutations and not other possible mechanisms (Perry et al. 2012). Our goal was to survey annual bluegrass populations previously reported resistant to PSII-inhibiting herbicides to evaluate the effect of their resistance mechanisms on susceptibility to amicarbazone, atrazine, and diuron; upon discovering a potentially unique population, our goal expanded to further

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Table 1. Annual bluegrass populations sources of origin, suspected herbicide resistances, and results of screening experiments.

Biotype	Location	Resistance suspected
Belair	Tupelo, MS	Simazine
Canton	Canton, MS	Simazine
Indianola	Indianola, MS	Simazine
Leflor	Leflore, MS	Simazine
Meadows	Tupelo, MS	Simazine
Meridian	Meridian, MS	Simazine
Red Bud	Kosciusko, MS	Simazine
Winona	Winona, MS	Simazine
DR3[a]	Oregon	Diuron
AU	Auburn, AL	None
GN[b]	Opelika, AL	Acetolactate synthase
TN[c]	Chattanooga, TN	Dinitroaniline
Weak bluegrass[d]		None
Supine bluegrass[d]		None

[a] Population DR3 was provided by Dr. Carol Mallory-Smith, Professor of Crop and Soil Science at Oregon State University (Corvallis, OR).

[b] GN was previously confirmed as resistant to acetolactate synthase-inhibiting herbicides (McElroy et al. 2013).

[c] TN was previously confirmed as resistant to the dinitroaniline family of mitotic-inhibiting herbicides (Cutulle et al. 2009).

[d] Populations were provided by Dr. B. Shaun Bushman of the U.S. Department of Agriculture, Forage and Range Research Laboratory (Logan, UT).

elucidate the role of nontarget mechanisms in resistance within that population.

Materials and Methods

Visual Control Ratings. A total of 14 populations from the genus *Poa* were grown and evaluated for resistance to PSII inhibitors, including 12 annual bluegrass populations and the annual bluegrass progenitor species weak bluegrass (*Poa infirma* Kunth) and supine bluegrass (*Poa supina* Schrad.) (Table 1) (Mao and Huff 2012). Atrazine- or amicarbazone-resistant populations or populations resistant to both atrazine and amicarbazone from Mississippi were acquired from Mississippi State University weed-seed storage for evaluation (Hutto et al. 2004; Perry et al. 2012). In addition, three susceptible populations from Auburn, AL (AU), Opelika, AL (GN), and Tennessee (TN) were evaluated as susceptible check populations. A suspected diuron-resistant population collected from seed-production fields in Oregon (DR3) was provided by Dr. Carol Mallory-Smith; no preexisting data were available regarding the level or type of resistance.

Annual bluegrass populations were evaluated for tolerance to two rates of amicarbazone, atrazine, and diuron in the greenhouse. Seeds were distributed into 10- by 10-cm plastic pots containing native Marvyn sandy loam (fine-loamy, kaolinitic, thermic, Typic Kanhapludult), pH 6.0, and 2.0% organic matter. Before germination, pots were irrigated two to three times daily to maintain adequate moisture for germination. Approximately 7 to 10 d after seeding, populations were thinned to five individual plants per pot. Plants were fertilized weekly with 5 g L^{-1} of 24–8–16 (N–P–K) fertilizer (Scotts Miracle-Gro Company, Marysville, OH). Herbicide treatments were initiated 5 wk after germination at a three- to five-tiller growth stage. Pots were arranged in a randomized complete-block design with four replications per treatment. Research was conducted in winter and spring 2012 to 2013. Studies were repeated twice in time.

Herbicides were applied using a single TeeJet TP8002EVS nozzle (Spraying Systems Co., Wheaton, IL) calibrated to apply at 280 L ha^{-1}. All treatments used Induce (Helena Chemical Company, Collierville, TN), a nonionic surfactant, at 0.25% v/v. Herbicide treatments were applied at the field rate and a higher check rate; they included amicarbazone (Arysta LifeScience Corporation, Cary, NC) applied at 0.35 and 0.7 kg ai ha^{-1}, atrazine (Syngenta Crop Protection Inc., Greensboro, NC), and diuron (Bayer Crop Science, Research Triangle Park, NC), both applied at 0.84 and 1.68 kg ai ha^{-1}.

Visual ratings for annual bluegrass control were taken 28 d after treatment (DAT). Control data were visually rated using a 0 to 100 scale, where 0 indicates no control, and 100 indicates complete plant death.

Sequencing of the *psbA* Gene. Considering that most of the populations evaluated had known or suspected resistance to PSII inhibitors, the *psbA* gene of all biotypes, weak bluegrass, and supine bluegrass were sequenced to identify the presence of previously reported mutations. To assess populations for known mutations conferring PSII-inhibitor resistance, a 637-base pair portion of the *psbA* gene was analyzed using the polymerase chain

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reaction (PCR) methods reported by Tian and Darmency (2006). With a Qiagen DNeasy Plant Kit (Qiagen, Valencia, CA), genomic DNA was extracted from samples of interest. Double-stranded DNA was then amplified by PCR in a 50-µl volume consisting of PCR buffer (New England BioLabs, Ipswich, MA), 200 mmol L^{-1} of deoxynucleotide triphosphate, 0.2 mmol L^{-1} of forward and reverse primers, 2 µl of Taq polymerase (New England BioLabs), and 2.5 µL of template DNA. The two primers used were psbAF (5′-ACTAGAAGTTAC CAAAGAACCATGC-3′) and psbAR (5′-GCATGGTTCTTTGGTAACTTCTAGT-3′), forward and reverse, respectively. Thermal cycling was performed with an initial denaturation at 95 C for 30 s, followed by 35 cycles of 95 C for 5 s, 58 C for 30 s, and 72 C for 1 min. PCR products were subsequently purified using a Qiaquick PCR purification kit (Qiagen). Sequencing was conducted at the Auburn University Genomics and Sequencing Laboratory under standard conditions with an ABI3100 sequencer (Applied Biosystems Inc., Foster City, CA). Sequences were aligned using CLC Workbench software (CLC Bio-Qiagen, Aarhus, Denmark), and nucleotide (blastn) and amino acid (blastx) evaluations were applied using the National Center for Biotechnology Information Basic Local Alignment Search Tool to align with nucleotide and transcribed amino acid sequences.

Atrazine Absorption, Translocation, and Metabolism. Research was conducted to evaluate the fate of ^{14}C-atrazine when applied to annual bluegrass leaves. Ideally, it was our goal to also evaluate fate of diuron and amicarbazone, however, we were not able to acquire ^{14}C-labeled versions of these materials. Annual bluegrass was established in pots with a 3.8-cm surface diameter and 20-cm depth in a greenhouse at the University of Georgia (Griffin, GA). Soil was 80 : 20 sand : peat moss (v/v) and the greenhouse temperature was set for 23/17 C (day/night). Pots were thinned to one plant during establishment. Shoots were trimmed weekly at a 5 cm height with shears and received irrigation as needed to prevent wilting. Grasses selected for treatments were at a four- to seven-tiller growth stage.

Plants were removed from pots and roots were rinsed free of soil. The grasses were then grown hydroponically in a 9-L plastic tank containing half-strength Hoagland solution (Hoagland and Arnon 1950). Roots were placed through 1-cm holes drilled approximately 3 cm apart in the lid of the container to facilitate submergence in solution. The tank was covered with aluminum foil, and an aquarium pump provided oxygen to the solution.

Plants were acclimated to hydroponic culture for 1 wk in the greenhouse and then placed in a growth chamber (Percival Scientific, Inc., Perry, IA) for 3 d. The chamber was set for 27/20 C (day/night) with 12-h photoperiods of 350 µmol m^{-2} s^{-1}. Plants were then removed from the hydroponic tank and placed in 5 ml plastic vials filled with 200 µl deionized water spiked with 3.7 kBq of ^{14}C-atrazine (160 mCi $mmol^{-1}$; ring labeled, 99% purity). Nonlabeled atrazine was added to the solution to simulate a 1.12 kg ai ha^{-1} surface application rate. Cotton balls were placed at the base of the shoots to facilitate placement of roots in treatment solution. After 4 h, 2 ml of tap water was applied to vials with a 5-ml pipette (Eppendorf Research Plus, Hamburg, Germany) to reduce moisture stress.

After 24 h, plants were placed back in the hydroponic tank with herbicide-free, half-strength Hoagland solution. Grasses were then harvested at 24, 72, or 168 h after treatment (HAT). Plants harvested at 24 HAT were not returned to the hydroponic tank. Roots were separated from shoots with shears and blotted dry with paper towels. For ^{14}C extraction, roots and shoots were minced separately and ground with 15 mL of methanol in a 50 ml plastic vial using a tissue homogenizer (FSH 125, Fisher Scientific LLC, Pittsburg, PA) for 30 s. The homogenizer was rinsed with an additional 5 ml of methanol that was collected in the vial. Samples were then sonicated for 1 h, centrifuged for 10 m, and the supernatant was transferred to separate tubes. This procedure was repeated with an additional 20 ml of methanol, and the supernatants were combined. A 4-ml aliquot was sampled from each tube, and radioactivity was quantified with liquid scintillation counting. Extraction efficiency averaged 87% (±0.9) after residue oxidation.

Supernatant was then evaporated in a hood on a heating block set for 50 C and then resuspended in 100 µl of methanol. Samples were spotted on 20- × 20-cm silica-gel plates that were developed to 16-cm in glass chambers using ethyl acetate : dichloromethane : acetic acid at 2 : 16 : 0.4 (v/v/v). Stock solutions of atrazine were developed on plates and identified at R_f 0.7. Metabolites were detected with a radiochromatogram scanner (BioScan AR-2000,

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Table 2. Annual bluegrass control after amicarbazone, atrazine, and diuron applications to triazine-resistant and triazine-susceptible populations, visual ratings 28 d after treatment.[a]

		Annual bluegrass control					
		%					
		Diuron		Atrazine		Amicarbazone	
Biotype	*psbA* amino acid substitution	0.84	1.68	0.84	1.68	0.35	0.7
		kg ai ha^{-1}					
Belair	Ser_{264} to Gly	73	90	0	0	0	0
Canton	Ser_{264} to Gly	98	99	3	0	0	0
Indianola	Ser_{264} to Gly	96	100	0	9	15	10
Leflor	Ser_{264} to Gly	63	77	0	36	10	36
Meadows	Ser_{264} to Gly	86	90	0	0	0	0
Meridian	Ser_{264} to Gly	99	100	4	14	13	0
Red Bud	Ser_{264} to Gly	98	100	0	0	0	46
Winona	Ser_{264} to Gly	99	100	0	0	0	0
DR3	—	6	17	19	32	20	33
AU	—	81	100	98	100	98	96
GN	—	100	100	91	100	100	100
TN	—	100	100	100	100	100	100
Weak bluegrass	—	75	98	71	50	50	98
Supine bluegrass	—	100	100	99	99	80	91
	$LSD_{0.05}$	26	20	23	31	27	29

[a] Data are pooled over two experimental repetitions conducted in Auburn, AL.

BioScan, Washington, DC) equipped with Laura chromatography data collection and analysis software (LabLogic Systems, Inc., Brandon, FL), which measured radioactivity.

The experimental design was completely randomized with four replications, and the experiment was repeated. Total radioactivity was calculated before application; absorption was calculated by dividing radioactivity recovered in the supernatant and the residue by the amount applied. Translocation was quantified by dividing the radioactivity recovered in shoots by the total ^{14}C absorbed. Parent herbicide recovery was calculated by multiplying the percentage of the total parent peak in roots and shoots by the ^{14}C extracted.

Statistical Analysis. Data were subjected to ANOVA with the general linear model procedure in SAS 9.1 (SAS Institute Inc., Cary, NC). Means were separated in PROC GLM, using Fisher's protected LSD test at the 0.05 probability level.

Results and Discussion

Visual Control Ratings. No significant differences between the two experimental runs were detected via ANOVA ($P = 0.1272$); therefore, data were combined for analysis. Based on visual ratings, all populations, with the exception of DR3, were susceptible to diuron (Table 2). DR3 resistance was observed across all herbicides and rates applied; relative to the nontreated, control of population DR3 was only slight to marginal, by both low and high rates of diuron, atrazine, and amicarbazone, ranging from 6 to 32% control. Low and high rates of all herbicides controlled susceptible populations AU, GN, and TN, ranging from 81 to 100% control. Regarding the ancestral species, weak bluegrass and supine bluegrass, control via diuron, atrazine, and amicarbazone was also achieved, although more efficiently for supine bluegrass (80–100% control) than it was for weak bluegrass (50 to 98% control). Although partial amicarbazone control was observed in Leflor and Red Bud populations at the highest rate of application, we attribute some of this variation to aphid damage that occurred to these populations while conducting these experiments. Inclusion of more-robust whole-plant data, such as biomass collection, would be beneficial to further delineate the impact of the herbicides because insect damage was difficult to separate from herbicide damage visually. Because of

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AU             AGCTGGTGTATTCGGCGGCTCCCTATTTAGTGCTATGCATGGTTCCTTGGTAACCTCTAG
Belair         AGCTGGTGTATTCGGCGGCTCCCTATTTAGTGCTATGCATGGTTCCTTGGTAACCTCTAG
DR3            AGCTGGTGTATTCGGCGGCTCCCTATTTAGTGCTATGCATGGTTCCTTGGTAACCTCTAG
                                                              I
Mutant         AGCTGGTGTATTCGGCGGCTCCCTATTTAGTGCTATGCATGGTTCCTTGATAACCTCTAG
Wild Type 471  AGCTGGTGTATTCGGCGGCTCCCTATTTAGTGCTATGCATGGTTCCTTGGTAACCTCTAG 530

AU             TTTGATCAGGGAAACTACTGAAAATGAATCTGCTAATGAGGGTTACAAATTTGGTCAAGA
Belair         TTTGATCAGGGAAACTACTGAAAATGAATCTGCTAATGAGGGTTACAAATTTGGTCAAGA
DR3            TTTGATCAGGGAAACTACTGAAAATGAATCTGCTAATGAGGGTTACAAATTTGGTCAAGA
Mutant         TTTGATCAGGGAAACTACTGAAAATGAATCTGCTAATGAGGGTTACAAATTTGGTCAAGA
Wild Type 531  TTTGATCAGGGAAACTACTGAAAATGAATCTGCTAATGAGGGTTACAAATTTGGTCAAGA 590

AU             GGAAGAAACTTATAATATTGTGGCTGCTCATGGTTATTTTGGCCGATTAATCTTCCAATA
Belair         GGAAGAAACTTATAATATTGTGGCTGCTCATGGTTATTTTGGCCGATTAATCTTCCAATA
DR3            GGAAGAAACTTATAATATTGTGGCTGCTCATGGTTATTTTGGCCGATTAATCTTCCAATA
Mutant         GGAAGAAACTTATAATATTGTGGCTGCTCATGGTTATTTTGGCCGATTAATCTTCCAATA
Wild Type 591  GGAAGAAACTTATAATATTGTGGCTGCTCATGGTTATTTTGGCCGATTAATCTTCCAATA 650

AU             TGCTAGTTTCAACAACTCTCGTTCTTTACACTTCTTCTTGGCTGCTTGGCCTGTAGTAGG
                    S
Belair         TGCTGGTTTCAACAACTCTCGTTCTTTACACTTCTTCTTGGCTGCTTGGCCTGTAGTAGG
DR3            TGCTAGTTTCAACAACTCTCGTTCTTTACACTTCTTCTTGGCTGCTTGGCCTGTAGTAGG
Mutant         TGCTAGTTTCAACAACTCTCGTTCTTTACACTTCTTCTTGGCTGCTTGGCCTGTAGTAGG
Wild Type 651  TGCTAGTTTCAACAACTCTCGTTCTTTACACTTCTTCTTGGCTGCTTGGCCTGTAGTAGG 710

AU             GATCTGGTTCACTGCTTTAGGTATTAGTACTATGGCTTTCAACCTAAATGGTTTCAATTT
Belair         GATCTGGTTCACTGCTTTAGGTATTAGTACTATGGCTTTCAACCTAAATGGCTTCAATTT
DR3            GATCTGGTTCACTGCTTTAGGTATTAGTACTATGGCTTTCAACCTAAATGGTTTCAATTT
Mutant         GATCTGGTTCACTGCTTTAGGTATTAGTACTATGGCTTTCAACCTAAATGGTTTCAATTT
Wild Type 711  GATCTGGTTCACTGCTTTAGGTATTAGTACTATGGCTTTCAACCTAAATGGTTTCAATTT 770

AU             CAACCAATCTGTAGTTGATAGTCAAGGTCGCGTTATTAATACTTGGGCTGATATCATCAA
Belair         CAACCAATCTGTAGTTGATAGTCAAGGTCGCGTTATTAATACTTGGGCTGATATCATCAA
DR3            CAACCAATCTGTAGTTGATAGTCAAGGTCGCGTTATTAATACTTGGGCTGATATCATCAA
Mutant         CAACCAATCTGTAGTTGATAGTCAAGGTCGCGTTATTAATACTTGGGCTGATATCATCAA
Wild Type 771  CAACCAATCTGTAGTTGATAGTCAAGGTCGCGTTATTAATACTTGGGCTGATATCATCAA 830
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Figure 1. Nucleotide sequences of Auburn, AL (AU), Belair, and Oregon (DR3) populations surrounding the reported Val_{219} to Ile and Ser_{264} to Gly mutation amino acid substitutions. Belair was presented as a representative for the group of Ser_{264} to Gly mutants with identical *psbA* gene sequences. Sequences were aligned with a Val_{219} to Ile mutant (National Center for Biotechnology Information [NCBI] accession number AF131886.1) and a wild-type annual bluegrass (NCBI accession number 131887.1). Amino acids substitutions are presented above the underlined altered codons.

the possibility of insecticide–herbicide interaction, only oil and soap insecticides were used in these experiments; this may have provided insufficient control when aphid populations increased. Thus, we attribute some of the injury to Leflor and Red Bud to insect injury. The remaining populations, Belair, Canton, Indianola, Meadows, Meridian, and Winona, were susceptible to diuron, but resistant to atrazine and amicarbazone, which further distinguishes our results for diuron-resistant population DR3.

Sequencing of *psbA* Gene. Following sequencing, nucleotide and transcribed amino acid sequences for the evaluated biotypes were aligned with the *psbA* chloroplast gene (Figure 1). A Ser_{264} to Gly point mutation was found in Belair, Canton, Indianola, Leflor, Meadows, Meridian, Red Bud, and Winona, which confers resistance to both atrazine and amicarbazone, as reported by Perry et al. (2012). No resistance to diuron was noticed from the Ser_{264} to Gly mutation, hence, the high control obtained from diuron applications to these populations.

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Table 3. Absorption, translocation, and metabolism of ^{14}C-atrazine in three annual bluegrass biotypes in two laboratory experiments, 2014, Griffin, GA.[a]

Biotype	Absorption	Translocation	Parent herbicide		
			Roots	Shoots	Total plant
	% of applied	% of absorbed	% of ^{14}C extracted		
AU	43	92	1	24	25
Belair	48	95	1	23	24
DR3	21	81	4	8	12
$LSD_{0.05}$	4	2	1	4	3
Harvest (HAT)					
24	39	84	4	26	30
72	37	91	1	15	15
168	36	92	1	15	16
$LSD_{0.05}$	NS	2	1	4	3
Biotype	*	*	*	*	*
Harvest	*	*	*	*	*
Biotype × harvest	NS	NS	NS	NS	NS

[a] Abbreviations: HAT, h after treatment; NS, not significant.

* Results were significant according to Fisher's protected LSD test at the 0.05 probability level.

Susceptible populations, including progenitor species weak bluegrass and supine bluegrass, as well as AU and TN did not have any *psbA* mutations related to herbicide resistance. Finally, no mutations were found in the *psbA* gene of the DR3 population, despite the increased tolerance toward PSII inhibitors exhibited by this population. In DR3, the Val_{219} to Ile mutation imparting resistance to diuron was also absent (Mengistu et al. 2000). The lack of mutations detected in the DR3 population necessitated further investigations toward nontarget-site mechanisms of resistance, such as absorption, translocation, and metabolism within annual bluegrass biotypes.

Atrazine Absorption, Translocation, and Metabolism. Three biotypes were selected to investigate in the plant fate of atrazine—AU (no mutation, susceptible to all), Belair (Ser_{264} to Gly, susceptible to diuron), and DR3 (no mutation, resistant to all). We hypothesized that DR3 would exhibit variation in absorption, translocation, or metabolism consistent with a nontarget-type resistance mechanism. Biotype-by-experiment interactions were not detected, and thus, results were combined over experimental runs. Biotype by harvest interactions were not detected for absorption, translocation, or metabolism (Table 3); therefore, data were combined for each biotype over harvest intervals. The AU and Belair biotypes absorbed 43 and 48% of the applied ^{14}C-atrazine, respectively. The DR3 biotype absorbed approximately 50% less ^{14}C-atrazine than AU and Belair did. Absorption was similar across harvests suggesting radioactivity was not lost after returning plants to the hydroponic tank at 24 HAT. Reduced absorption of DR3 agrees with findings of Singh et al. (2015) showing increased metabolism coupled with reduced absorption results in higher simazine tolerance for zoysiagrass (*Zoysia japonica* Steud.) and bermudagrass when compared with the more sensitive seashore paspalum (*Paspalum vaginatum* Sw.).

Annual bluegrass translocated 84% of the absorbed ^{14}C to shoots at 24 HAT and increased to an average 92% at 72 and 168 HAT. The DR3 biotype translocated approximately 12% less ^{14}C to shoots than the other biotypes had. There was no meaningful difference in radioactivity translocation between AU and Belair.

Enhanced herbicide metabolism increases resistance to chlorotoluron, simazine, metribuzin, and other PSII-inhibiting herbicides in rigid ryegrass (Burnet et al. 1993a; Preston and Powles 1997). Analogously, metabolism was noted as the physiological basis for variable sensitivity of different cultivars of bahiagrass (*Paspalum notatum* Flüggé) to atrazine (Smith 1983). Two metabolites of ^{14}C-atrazine were detected in roots of all annual bluegrass biotypes at R_f 0.1 and 0.5 (data not

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shown). These metabolites plus a third at R_f 0.3 were detected in shoots at all harvests. Metabolism increased from 24 to 72 HAT in all biotypes. Differences in parent herbicide recovery were not detected between AU and Belair because 25% of the extracted ^{14}C was the parent herbicide. However, the DR3 biotype averaged two times less total parent herbicide from these biotypes. The amount of parent atrazine recovered in shoots was three times lower in DR3 than it was in AU and Belair. Nonmetabolized atrazine accounted for 1% of the extracted ^{14}C in roots of AU and Belair but DR3 was four times greater.

Nontarget mechanisms of tolerance to PSII herbicides have been previously reported. Roeth and Lavy (1971) found sorghum [*Sorghum bicolor* (L.) Moench ssp. *bicolor*] and sudangrass [*Sorghum sudanense* (Piper) Stapf] absorbed two to three times more ^{14}C-atrazine than a more tolerant species, corn (*Zea mays* L.). Similarly, Davis et al. (1965) reported that soybean [*Glycine max* (L.) Merr.] absorbed more root-applied ^{14}C-atrazine after 24 h than two more-tolerant species, corn and cotton (*Gossypium hirsutum* L.). Evaluation of herbicide interception via root-applied amicarbazone and atrazine revealed that large crabgrass [*Digitaria sanguinalis* (L.) Scop.] and velvetleaf were similarly affected by the two herbicides; however, corn was significantly less affected by root-applied atrazine (Dayan et al. 2009).

Nontarget site–based triazine resistance in Illinois populations of tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] indicates the potential for nontarget-site forms of PSII-inhibiting herbicide resistance in other weed species (Patzoldt et al. 2003). Two induced mutants of metribuzin-resistant narrowleaf lupin (*Lupinus angustifolius* L.) also lacked *psbA* target site mutations, thus demonstrating nontarget site–based resistance in another dicotyledonous weed (Pan et al. 2012). Following early inhibition of photosynthesis, metribuzin-resistant mutants recovered photosynthetic capabilities 2.5 DAT; this, when combined with further investigations of cytochrome P450 monooxygenase inhibitors, demonstrated the involvement of cytochrome P450 enzymes in the two populations' metribuzin resistance.

In grass species, nontarget PSII-inhibiting herbicide resistance has been reported in rigid ryegrass and blackgrass (*Alopecurus myosuroides* Huds.) (Burnet et al. 1993a; Hall et al. 1995; Menendez et al. 1994; Preston and Powles 1997). Light-induced chlorotoluron metabolism increased the degradation of the substituted urea in a resistant population of rigid ryegrass (Preston and Powles 1997). Similarly, increased metabolism is indicated as a possible source of resistance in a blackgrass population, which absorbed and translocated ^{14}C-chlorotoluron at normal rates for both resistant and susceptible populations (Menendez et al. 1994).

Despite lacking any known mutations in the *psbA* gene previously linked to resistance in annual bluegrass, population DR3 is resistant to three PSII inhibitors, including amicarbazone, atrazine, and diuron. Compared with PSII-resistant populations with known Ser_{264} to Gly mutations, DR3 is similarly resistant to the selected herbicides based on visual ratings. Further evaluation of herbicide movement throughout the DR3 population revealed a potentially unique mechanism of resistance in this annual bluegrass biotype, with lower atrazine absorption and translocation indicated by considerably lower levels of herbicide present in the final biomass. Warranting further examination, DR3 exhibits nontarget-site resistance; decreased herbicide absorption and translocation are indicated as physiological factors involved in the loss of herbicide efficacy when applied to DR3. Tolerance and resistance linked to reduced absorption and increased metabolism may lead to distinct occurrences of field resistance in weed populations, which may ultimately pose an increasingly difficult situation for resistance management.

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