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RESEARCH PAPER

Glyphosate resistance in common ragweed (*Ambrosia artemisiifolia* L.) from Mississippi, USA

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Glyphosate is one of the most commonly used broad-spectrum herbicides over the last 40 years. Due to the widespread adoption of glyphosate-resistant (GR) crop technology, especially corn, cotton and soybean, several weed species have evolved resistance to this herbicide. Research was conducted to confirm and characterize the magnitude and mechanism of glyphosate resistance in two GR common ragweed (*Ambrosia artemisiifolia* L.) biotypes from Mississippi, USA. A glyphosate-susceptible (GS) biotype was included for comparison. The effective glyphosate dose to reduce the growth of the treated plants by 50% for the GR1, GR2 and GS biotypes was 0.58, 0.46 and 0.11 kg ae ha^{-1}, respectively, indicating that the level of resistance was five and fourfold that of the GS biotype for GR1 and GR2, respectively. Studies using ^{14}C-glyphosate have not indicated any difference in its absorption between the biotypes, but the GR1 and GR2 biotypes translocated more ^{14}C-glyphosate, compared to the GS biotype. This difference in translocation within resistant biotypes is unique. There was no amino acid substitution at codon 106 that was detected by the 5-enolpyruvylshikimate-3-phosphate synthase gene sequence analysis of the resistant and susceptible biotypes. Therefore, the mechanism of resistance to glyphosate in common ragweed biotypes from Mississippi is not related to a target site mutation or reduced absorption and/or translocation of glyphosate.

Keywords: absorption, EPSPS, herbicide resistance, mutation, translocation.

Common ragweed is a summer annual weed in several row crops of the south-eastern USA, including Mississippi. The ability of common ragweed to grow in moist, low-fertility soil has caused its occurrence in non-croplands, pastures, ditches, creek banks and roadsides (Royer & Dickinson 2004; Jordan *et al.* 2014). Before the introduction of glyphosate-resistant (GR) crops in the mid-1990s (Woodburn 2000), acetolactate synthase (ALS) and protoporphyrinogen oxidase (PPO) inhibitors were the main tools for controlling common ragweed across the USA and Canada (Rousonelos *et al.* 2012). However, the efficacy of ALS- and PPO-inhibiting herbicides was short-lived and common ragweed evolved resistance to these modes of action in less than a decade (Schultz *et al.* 2000; Moreira *et al.* 2006; Heap 2016).

Glyphosate has been used extensively across the world in both crop and non-crop lands since its commercialization in 1974 (Dyer 1994). It potently inhibits the plastidic enzyme 5-enolpyruvylshikimate-3-phosphate synthase

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(EPSPS) (EC 2.5.1.19) in the shikimate pathway (Amrhein *et al.* 1980), which synthesizes many aromatic amino acids and phenolic complexes (Herrmann & Weaver 1999). The overreliance on glyphosate and the widespread adoption of GR crops around the world has resulted in the evolution of GR weed biotypes (Powles & Preston 2006). To date, 35 weed species are reported to be resistant to glyphosate (Heap 2016), including common ragweed. The first report of GR common ragweed was from Missouri, USA, in 2004 (Pollard *et al.* 2004). Subsequently, in the same year, glyphosate failed to control a common ragweed population in an Arkansas soybean (*Glycine max* L. Merr.) field (Brewer & Oliver 2009). To date, GR common ragweed has been identified in 14 states of the USA and Ontario Province in Canada (Heap 2016). More recently, two common ragweed biotypes (GR1 and GR2) from northeastern Mississippi, USA, have survived glyphosate applications at the labeled field rate (0.84 kg ae ha^{-1}).

The objectives of this research were to: (i) estimate the level of resistance to glyphosate in common ragweed biotypes: and (ii) characterize the molecular and physiological mechanisms of resistance in the resistant biotypes. Dose–response, ^{14}C-glyphosate absorption and translocation and EPSPS gene sequence analysis studies were conducted.

METHODS

Seed collection, storage, germination, planting, growth and herbicide treatment conditions

Inflorescences that contained seed from 10 mature common ragweed plants that were suspected to be resistant to glyphosate were collected from a field near Saltillo, Lee County, Mississippi, USA, in 2014. The field had been under continuous GR soybean for at least 6 years. The bulked seed was air-dried for 1 week in a greenhouse at the Jamie Whitten Delta States Research Center of the United States Department of Agriculture–Agricultural Research Service in Stoneville, Mississippi, USA, that was set to 25/20 ± 3°C° day/night temperature and a 13 h photoperiod that was provided by high-pressure sodium lights (400 μmol m^{-2} s^{-1}). The seed then was cleaned and stored in a cold room until further use. A susceptible biotype (GS) that was provided by M. Christoffers of North Dakota State University, Fargo, ND, USA, was included for comparison. The GR and GS seeds were sown individually in plastic trays (50 cm × 20 cm × 6 cm) that contained a commercial LC1 potting mix (Sun Gro Horticulture Distribution Inc., Bellevue, WA, USA). They were watered and incubated at 4°C for 7 days. Subsequently, the trays were relocated into the above-mentioned greenhouse. The emerged common ragweed plants were transplanted into 15 cm diameter plastic pots at the two-leaf stage and used in the experiments that are described below. Following transplanting, the plants were watered as needed and fertilized 14 days later with a water-soluble fertilizer (Miracle-Gro, Scotts Miracle-Gro Products, Inc., Marysville, OH, USA). All the herbicide treatments were applied at the three-leaf (node) stage (10 cm tall) plants by using an air-pressurized indoor spray chamber (DeVries Manufacturing Company, Hollandale, MN, USA) that was equipped with a nozzle mounted with 8002E flat-fan tips (Spraying Systems Company, Wheaton, IL, USA) that delivered 140 L ha^{-1} at 280 kPa.

Screening with a discriminating glyphosate dose

In the preliminary resistance screening studies, 100 plants that had been raised from the seed of the suspected resistant plants were treated with a 0.84 kg ae ha^{-1} rate of glyphosate (potassium salt, Roundup WeatherMAX®; Monsanto Company, St. Louis, MO, USA) (data not shown). Two plants that had survived the glyphosate treatment by 3 weeks after treatment (WAT) were taken to maturity to produce seed. These two plants were designated as biotypes GR1 and GR2. Additional screening experiments with the seed from the GR1 and GR2 plants indicated that all the plants survived a glyphosate treatment of 0.84 kg ae ha^{-1} (data not shown). This second-generation seed was used in all subsequent studies.

Glyphosate dose–response

The plants of the resistant (GR1 and GR2) and susceptible (GS) biotypes were treated with 0, 0.21, 0.42, 0.84, 1.68, 3.36 and 6.72 kg ae ha^{-1} and 0, 0.026, 0.053, 0.11, 0.21, 0.42 and 0.84 kg ae ha^{-1} of glyphosate, respectively. A visual assessment of the plant control percentage on a scale of 0 ("no injury") to 100 ("plant death") was recorded 3 WAT. There were three replications per treatment and the experiment was repeated.

^{14}C-glyphosate absorption, translocation and phosphorimaging

Two-to-three-leaf stage common ragweed plants were transferred from the greenhouse to a growth chamber 7 days prior to the ^{14}C-glyphosate application for acclimatization. The growth chamber was maintained at 25/20°C with a 13 h photoperiod (300 μmol m^{-2} s^{-1}) that was provided by fluorescent and incandescent

bulbs. The plants were left in the growth chamber until harvest. All the plants were treated with the labeled field rate of glyphosate, as described before, except that one of the second or third fully expanded leaf was covered with a waterproof paper sleeve for follow-up ^{14}C-glyphosate treatment. An overspray with unradioactive glyphosate (or the herbicide under investigation) has been reported before (Lorraine-Colwill *et al.* 2003; Nandula *et al.* 2013), while it also has not been practiced elsewhere (Koger & Reddy 2005).

A solution that contained glyphosate at a final concentration of 0.84 kg ae ha^{-1} in 140 L ha^{-1} was made by using ^{14}C-glyphosate, a commercial potassium salt formulation of glyphosate (^{14}C-methyl labeled with 2.0 GBq mmol^{-1} specific activity; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA). A 10 μL volume of the solution was applied to the adaxial surface of the covered second fully expanded leaf in the form of tiny droplets with a micro applicator. Each plant received ~7.3 kBq of ^{14}C-glyphosate in a total volume of 10 μL. The plants were harvested at 1, 4, 24, 48 and 96 h after treatment (HAT). Thereafter, standard procedures to measure the absorption and translocation of ^{14}C- glyphosate in common ragweed plants from all biotypes were followed, as described below.

At each harvest, the treated leaf was removed and rinsed in 10 mL of 10% methanol for 20 s to remove the unabsorbed ^{14}C-glyphosate from 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 separate 10 mL scintillation cocktail (Ecolume; ICN, Costa Mesa, CA, USA) volumes to measure the unabsorbed ^{14}C-glyphosate. After removing the treated leaf, each plant was divided further into the shoot above the treated leaf, the shoot below the treated leaf, and the roots for measuring translocation. The above four plant parts were wrapped individually in a single layer of tissue paper (Kimwipes; Kimberly-Clark Corporation, Roswell, GA, USA), placed in a glass vial and oven-dried at 60 C° for 48 h. The oven-dried plant samples were combusted in a biological oxidizer (Packard Instruments Company, Downers Grove, IL, USA) and the evolved $^{14}CO_2$ was trapped in 10 mL of Carbosorb E (Packard BioScience Company, Meridian, CT, USA) and 10 mL of Permaflour E+ (Packard BioScience). The level of radioactivity from the leaf washes and oxidations was quantified by using liquid scintillation spectrometry (Packard Tri-Carb 2100TR; Packard Instrument Company, IL, USA). The average recovery of the applied ^{14}C-glyphosate was 97%, based on the sum of the radioactivity that was measured in all the plant parts (absorption, expressed as a percentage of the applied ^{14}C) and leaf washes. The total level of radioactivity that was recovered in all the plant parts, except the treated leaf, was designated as the translocated ^{14}C and expressed as a percentage of the absorbed ^{14}C-glyphosate. There were five replications per treatment for all biotypes.

A separate set of plants of all three biotypes was treated with ^{14}C-glyphosate, as described before. At 24 and 48 HAT, the treated leaves from the plants were removed to wash off any unabsorbed radioactivity and they were set aside. The remaining above-ground part of the plant was excised from the roots and mounted on a 27 cm × 21.25 cm piece of plain white paper. The shoot parts were spread evenly and kept in place with thin strips of clear office tape. Care was taken to avoid contact of the washed treated leaf with the other parts of the plant. The roots were gently rinsed with water to remove the soil, blotted dry with paper towels and mounted on a separate sheet, as with the shoot. The mounted plant parts were pressed between one or more layers of newspaper and bound 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 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 cm × 40 cm exposure cassette (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA) and brought into contact with a storage phosphor screen (BAS IPSR 2025 E; GE Healthcare Bio-Sciences Corporation) under diffused lighting. The apparatus was placed in a dark cabinet for 24 h. A phosphorimager (Typhoon FLA 7000; GE Healthcare) was used to detect the distribution of ^{14}C-glyphosate and to develop an image. There were two replications per harvest time per biotype and the experiment was repeated.

Sequence analysis of EPSPS

A partial *epsps* gene of the common ragweed biotypes was amplified by using a primer pair (AtF1: 5′-ACA TGCTTGGGGCTCTAAGAA-3′ and AtR1: 5′-TTG AATTACCACCAGCAGCGGT-3′) that was designed in the authors' previous research on GR giant ragweed (Nandula *et al.* 2015). The primers were intended to amplify the regions covering the Gly_{101}, Thr_{102} and Pro_{106} codons in the GS and GR biotypes (Sammons & Gaines 2014). The RNA was isolated from the fresh frozen shoot tissues from plants at the three-to-four leaf stage by using a RNeasy Plant Mini

Kit (Qiagen, Venlo, The Netherlands). The RNA's integrity was checked by using 1% v/v formaldehyde agarose gel electrophoresis at 90 V alongside a RiboRuler high-range RNA ladder (Fermentas, Hanover, MD, USA). The cDNA was synthesized for all the common ragweed biotypes by using the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific, Waltham, MA, USA).

Each 25 μL polymerase chain reaction (PCR) reaction contained 12.5 μL of GoTaq Green Master Mix (Promega, Madison, WI, USA), 20 ng μL^{-1} of single-strand cDNA, 0.5 μL of each primer (19 μM) and 9.5 μL of nuclease-free water. A Bio-Rad T100 (Hercules, CA, USA) thermocycler was programed based on Nandula *et al.* (2015), as follows: 2 min initial denaturation at 94°C, followed by 35 cycles of 30 s at 95°C, 20 s at 56°C and 1 min at 72°C, including a final extension of 5 min at 72°C. The PCR products were fractioned by electrophoresis on 1.5% v/v agarose gel in 1 × TBE buffer alongside a GeneRuler™ 100 bp Plus ladder (Fisher Scientific–USA, Pittsburgh, PA, USA) at 90 V. The agarose gels were stained in 0.5 μg mol L^{-1} of ethidium bromide solution and imaged by using the GENi2 gel documentation system. Amplicons at ~200 bp for the GR and GS biotypes were purified by using a QIAquick PCR Purification Kit (Qiagen) at the DNA-sequencing facilities at Eurofins Genomics (Louisville, KY, USA). The sequences of all the biotypes were edited in BioEdit, a biological sequence alignment editor (Ibis Biosciences, Carlsbad, CA, USA), aligned and compared with Clustal Omega (European Bioinformatics Institute, Hinxton, UK).

Statistical analysis

All the experiments were conducted by using a completely randomized design. The data from all the experiments, with the exception of the EPSPS sequence analysis, were analyzed by ANOVA via the PROC GLM statement using SAS software (v. 9.2, SAS Institute, Inc., Cary, NC, USA). The data from the repeated dose–response experiments were pooled due to a non-significant experimental effect. A non-linear regression analysis was applied to fit a sigmoidal log-logistic curve of the form:

$$y = a/(1 + exp[-(x - x0)/b]),$$

where a is an asymptote, x and $x0$ are the upper and lower response limits, with the latter approaching 0, and b is the slope of the curve around $x0$ to relate the effect of the glyphosate dose on common ragweed control and the HAT on ^{14}C-glyphosate absorption and translocation. The equation parameters were computed by using SIGMAPLOT (v. 11.0, Systat Software, Inc., San Jose, CA, USA). The treatment means in selected experiments were separated by using Fisher's protected Least Significant Difference test at $P = 0.05$.

RESULTS AND DISCUSSION

Glyphosate dose–response

The response of the common ragweed biotypes to the glyphosate dose is presented in Fig. 1. The ED_{50} (the dose required to reduce plant growth by 50%) values for the GR1, GR2 and GS biotypes, based on the percentage control, were 0.58, 0.46 and 0.11 kg ae ha^{-1} of glyphosate, respectively. The resistance index that was calculated from the above ED_{50} values indicated that the GR1 and GR2 biotypes were five and fourfold, respectively, more resistant to glyphosate than the GS biotype. The resistance levels that are reported here are lower than those that have been documented previously. Pollard *et al.* (2004) reported a 9.6-fold and Brewer and Oliver (2009) found a 10- to 22-fold resistance level in Missouri and Arkansas common ragweed populations, respectively. Recently, a Nebraska population expressed an eight- to 19-fold resistance level to glyphosate (Ganie *et al.* 2016). The GR1 and GR2 biotypes survived glyphosate for ≤3.36 kg ae ha^{-1}. Phenotypically, the resistant plants exhibited curling of the leaf tips, with the mature leaves being unaffected by glyphosate at 0.84–3.36 kg ae ha^{-1}. Ganie *et al.* (2016) reported similar symptomatology.

^{14}C-glyphosate absorption, translocation and phosphorimaging

The absorption pattern of ^{14}C-glyphosate in the GR1, GR2 and GS biotypes was similar throughout the time course of the experiment, with a maximum of 42, 43, and 40% of the applied ^{14}C-glyphosate being reached at 96 HAT for the GR1, GR2 and GS biotypes, respectively (Fig. 2). Previous reports have documented analogous levels of ^{14}C-glyphosate absorption between the resistant and the susceptible plants in common ragweed (Brewer & Oliver 2009; Ganie *et al.* 2016) and giant ragweed (*Ambrosia trifida* L.) (Nandula *et al.* 2015).

The translocation pattern of ^{14}C-glyphosate was different between the resistant and the susceptible biotypes, with one or both resistant biotypes accumulating more glyphosate than the GS biotype at 24, 48 and 96 HAT (Fig. 3). The GR2 biotype translocated 51%

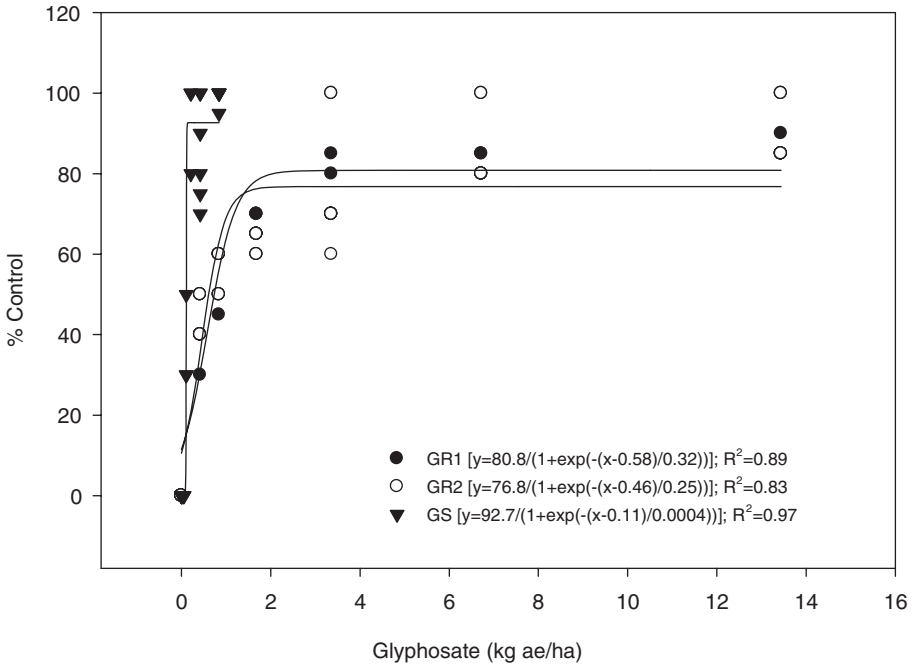

Fig. 1. Percentage control of the glyphosate-resistant (GR1 and GR2) and -susceptible (GS) common ragweed biotypes 3 weeks after treatment. (●), GR1 ($y = 80.8/[1 + \exp(-[x - 0.58]/0.32)]$), $R^2 = 0.89$; (○), GR2 ($y = 76.8/[1 + \exp(-[x - 0.46]/0.25)]$), $R^2 = 0.83$; (▼), GS ($y = 92.7/[1 + \exp(-[x - 0.11]/0.0004)]$), $R^2 = 0.97$.

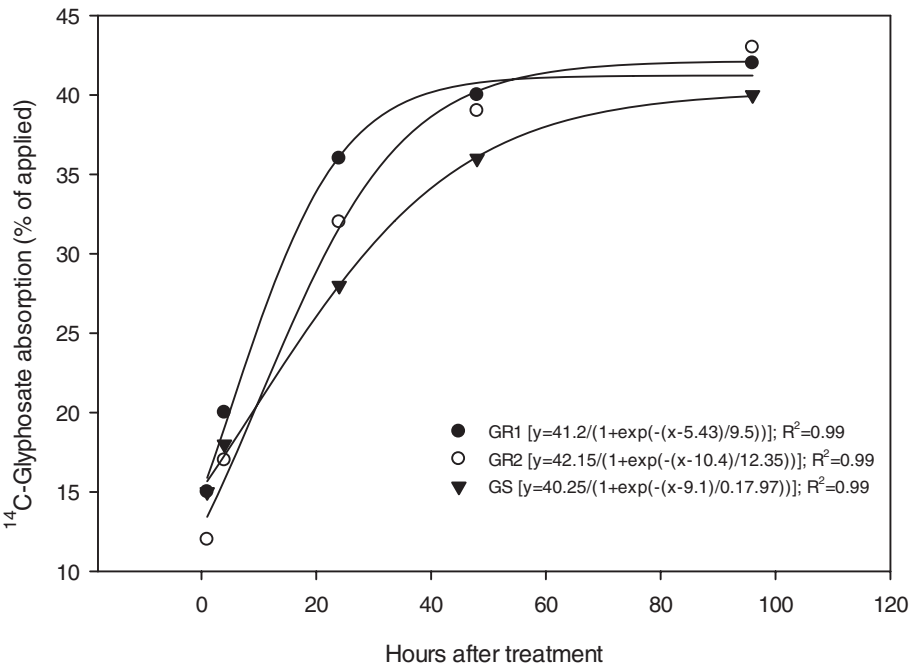

Fig. 2. Absorption of ^{14}C-glyphosate in the resistant (GR1 and GR2) and the susceptible (GS) common ragweed biotypes. (●), GR1 ($y = 41.2/[1 + \exp(-[x - 5.43]/9.5)]$), $R^2 = 0.99$; (○), GR2 ($y = 42.15/[1 + \exp(-[x - 10.4]/12.35)]$), $R^2 = 0.99$; (▼), GS ($y = 40.25/[1 + \exp(-[x - 9.1]/17.97)]$), $R^2 = 0.99$.

of the absorbed ^{14}C-glyphosate away from the treated leaf at 24 HAT, which was more than those of the GR1 (35% of the absorbed ^{14}C-glyphosate) and GS (33% of the absorbed ^{14}C-glyphosate) biotypes. At 48 and 96 HAT, both the GR1 (53 and 63% of the absorbed ^{14}C-glyphosate, respectively) and GR2 (54 and 65% of the absorbed ^{14}C-glyphosate, respectively) biotypes translocated higher levels of ^{14}C-glyphosate, compared to the GS (33 and 32% of the absorbed ^{14}C-glyphosate, respectively) biotype.

The above pattern is not indicative of a reduced translocation type of glyphosate resistance mechanism, nor similar to a pattern of a lack of differential translocation in a resistant common ragweed population (Brewer & Oliver 2009; Ganie *et al.* 2016). The lower levels of glyphosate translocation out of the treated leaf

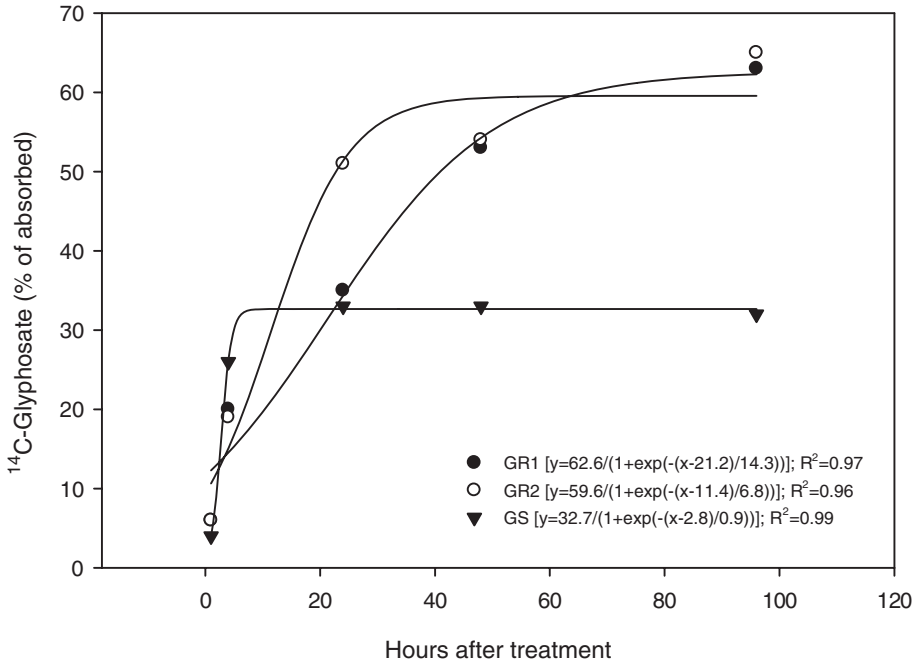

Fig. 3. Translocation of ^{14}C-glyphosate in the resistant (GR1 and GR2) and the susceptible (GS) common ragweed biotypes. The total amount of radioactivity that was recovered in all the plant parts, except the treated leaf, was designated as the translocated ^{14}C-glyphosate. (●), GR1 ($y = 62.6/[1 + \exp(-[x - 21.2]/14.3)]$), $R^2 = 0.97$; (○), GR2 ($y = 59.6/[1 + \exp(-[x - 11.4]/6.8)]$), $R^2 = 0.96$; (▼), GS ($y = 32.7/[1 + \exp(-[x - 2.8]/0.9)]$), $R^2 = 0.99$.

in the GS biotype, compared to one or both resistant biotypes at ≥24 HAT, could most likely be attributed to the inhibitory effect of the overspray with a 0.84 kg ae ha^{-1} rate of glyphosate. A susceptibility to glyphosate could have started to severely inhibit the GS plants at an increased rate at 24 HAT, thereby reducing the translocation from the source (treated leaf) to the sink (growing points, roots etc.). The movement of a herbicide such as glyphosate, which simulates photosynthate transport, to metabolic sinks in a sensitive plant is restricted because of the saturation of the sinks and the establishment of a reverse concentration gradient from the sink to the source leaves when applied at a labeled rate (Shaner 2009; Kniss *et al.* 2011). As a result of an appreciable increase in the amount of translocation of glyphosate in the GR1 and GR2 biotypes, the glyphosate translocation model that was proposed by Shaner (2009), which purports the existence of a barrier at the cellular level that prevents glyphosate loading into the phloem, does not relate to the resistant biotypes. Conversely, it is plausible to conceive the presence of a mechanism of avoidance or reduced glyphosate accumulation in the mesophyll cells and phloem, respectively. The glyphosate in the GR1 and GR2 plants essentially could be loaded into a vacuole via a system akin to the sequestration mechanism that was described for horseweed (*Conyza canadensis* L. Cronq.) (Ge *et al.* 2010) and ryegrass (*Lolium* spp.) (Ge *et al.* 2012), resulting in the phenotypic response mentioned earlier.

The distribution of the absorbed ^{14}C-glyphosate in the resistant and the susceptible biotypes is summarized in Table 1. The quantity of ^{14}C-glyphosate that accumulated in the treated leaf was higher in the GS biotype than the GR2 biotype (49% of the absorbed ^{14}C-glyphosate), but similar to the GR1 biotype (65% of the absorbed ^{14}C-glyphosate) at 24 HAT. In addition, the treated leaf of GS (67–68% of the absorbed ^{14}C-glyphosate) had more ^{14}C-glyphosate remaining than in both resistant biotypes (35–47% of the absorbed ^{14}C-glyphosate) at 48 and 96 HAT. The level of ^{14}C-glyphosate that translocated to the shoot above the treated leaf was lower in the GS biotype (10% of the absorbed ^{14}C-glyphosate at 96 HAT) than in the resistant biotypes (22% of the absorbed ^{14}C-glyphosate). The levels of ^{14}C-glyphosate that were retained in the shoot below the treated leaf by the GS biotype were lower than those of the resistant biotypes at 1 and 48 HAT, but higher by 96 HAT. In the roots, the distribution of the translocated ^{14}C-glyphosate was lower in the GS biotype, compared to the GR1 and GR2 biotypes at 4 HAT and all subsequent harvest timings. Overall, the distribution data reflect the translocation data, in that the GR1 and GR2 biotypes translocated more ^{14}C-glyphosate as time progressed after the treatment with glyphosate, being physiologically unaffected related to a lack of glyphosate toxicity. Conversely, the GS biotype was being suppressed progressively by the systemic action of glyphosate, resulting in a lack of translocation of additional glyphosate to other parts of the plant and leading to a feedback inhibition of the source–sink concentration gradient.

Table 1. Distribution[†] of ^{14}C-glyphosate in glyphosate-resistant and -susceptible common ragweed biotypes

Biotype	Number of h after treatment																			
	1	4	24	48	96	1	4	24	48	96	1	4	24	48	96	1	4	24	48	96
	Absorbed 14C-glyphosate (%)																			
	TL					SATL					SBTL					Root				
GR1	94	80	65	47	37	1	7	11	14	22	3	5	5	8	7	2	8	19	31	34
GR2	94	81	49	46	35	3	8	16	16	22	2	5	7	8	8	1	6	28	30	35
GS	96	74	67	67	68	1	19	14	13	10	1	3	7	5	12	2	4	12	15	10
LSD (0.05)[‡]	NS	NS	9	5	5	NS	NS	NS	NS	5	1	NS	NS	2	2	NS	3	8	5	6

[†] Distribution represents the partitioning of the absorbed ^{14}C-glyphosate between the treated leaf, shoot above the treated leaf, shoot below the treated leaf and the root). [‡] Fisher's protected Least Significant Difference test (0.05): A number indicates significance at the 5% level of probability and "NS" indicates no significant difference between the means within the same column. GR, glyphosate-resistant; GS, glyphosate-susceptible; SATL, shoot above the treated leaf; SBTL, shoot below the treated leaf; TL, treated leaf.

The phosphorimaging results (Fig. 4) mimicked the translocation results. The intensity of the shoot phosphorimage of the GR1 shoot (Fig. 4b, top panel) was denser, compared to the corresponding phosphorimages of the GR2 (Fig. 4b, middle panel) and GS (Fig. 4b, lower panel) biotypes at 24 HAT, reflecting a greater level of translocation in the GR1 biotype. Likewise, the GR1 (Fig. 4d, top panel) and GR2 (Fig. 4d, middle panel) shoot phosphorimages were more intense than that of the GS biotype (Fig. 4d, lower panel) at

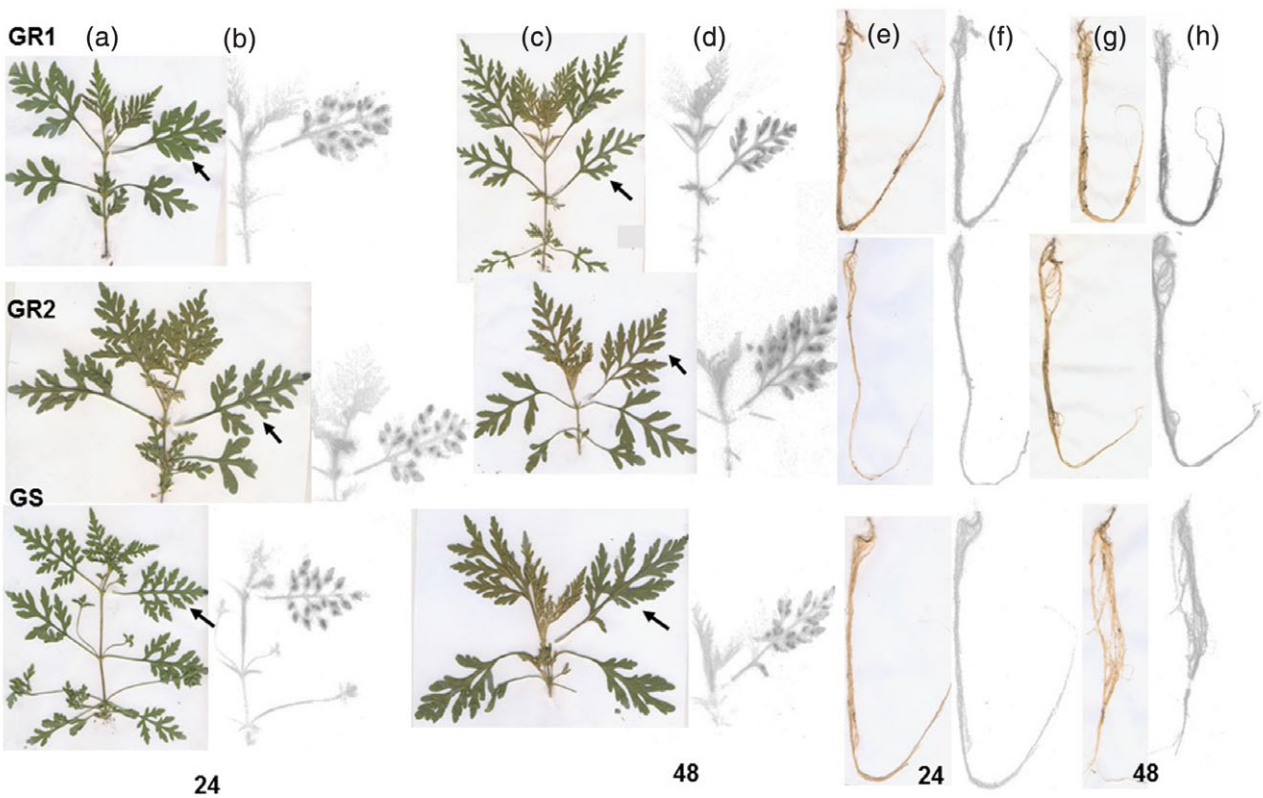

Fig. 4. Plants ([a, c] shoot; [e, g] root) and the corresponding phosphorimages ([b, d] shoot; [f, h] root) of the resistant GR1 (top panel), resistant GR2 (middle panel) and susceptible GS (lower panel) common ragweed biotypes. The arrows indicate the treated leaf.

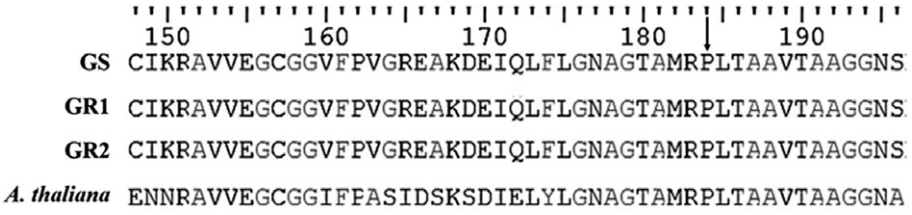

Fig. 5. Alignment of the plastidic enzyme 5-enolpyruvylshikimate-3-phosphate synthase amino acid sequences of *Arabidopsis thaliana* for the susceptible (GS) and the resistant (GR1 and GR2) common ragweed biotypes. The black arrow indicates the location of the Pro_{106} codon.

48 HAT. In general, the intensity of the root phosphorimages of the GR1 (Fig. 4f and h, top panel) and GR2 (Fig. 4f and h, middle panel) biotypes was darker than that of the GS biotype (Fig. 4f and h, lower panel) at 24 and 48 HAT.

Sequence analysis of EPSPS

The sequencing results of the common ragweed biotypes that were obtained via the Sanger sequencing method aligned with *Arabidopsis thaliana* (GenBank accession number CAA29828.1). There was no missense mutation detected within the EPSPS gene of the resistant biotypes (Fig. 5). Based on the chromatographic data, a single peak at the Pro_{106} codon of the resistant biotypes' sequences was observed that confirms homozygosity at this region. This result indicates that glyphosate resistance in the GR1 and GR2 common ragweed biotypes is not related to target site mutation in the conserved region of the EPSPS gene and that a mechanism other than amino acid substitution is responsible for this resistance.

In summary, neither of the GR common ragweed biotypes from Mississippi, USA, exhibit differential absorption and/or translocation of glyphosate nor do they possess a target site mutation at the Pro_{106} locus of their respective EPSPS. Amplification of the EPSPS gene, as found in Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Gaines *et al.* 2010), was not investigated in the Mississippi biotypes, but GR common ragweed from Nebraska did not reveal gene amplification as a resistance mechanism (Ganie *et al.* 2016). Further research may consider glyphosate metabolism (although not implicated as a mechanism of glyphosate resistance in GR weeds to date) and sequestration as possible resistance mechanisms.

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