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ARTICLE

Investigating the mechanism of glyphosate resistance in a common ragweed (*Ambrosia artemisiifolia* L.) biotype from Nebraska

Z.A. Ganie, M. Jugulam, V.K. Varanasi, and A.J. Jhala

Abstract: Common ragweed is a weed in the midwestern United States and eastern Canada that is difficult to control due to the evolution of an important resistance to multiple herbicides including glyphosate. Recently, a common ragweed biotype with 19-fold glyphosate resistance was confirmed in Nebraska. The objective of this study was to determine the mechanism of glyphosate resistance in a common ragweed biotype from Nebraska. Both target site and non-target site based mechanisms of glyphosate resistance were investigated using glyphosate-resistant (GR) and known glyphosate-susceptible (GS) common ragweed biotypes. A lower amount of shikimate was accumulated in the GR ($\leq$65 μg mL^{-1}) compared with the GS ($\geq$80 μg mL^{-1}) biotype at all glyphosate concentrations tested. Sequencing of the conserved region of the *EPSPS* gene revealed no mutations at the Thr_{102} or Pro_{106} residues and no variation in *EPSPS* copy number was detected. A higher translocation of ^{14}C-glyphosate in the GR compared with the GS biotype was found, although there was no difference in the amount of ^{14}C-glyphosate absorbed. Nonetheless, analysis of ^{14}C-glyphosate absorption or translocation data using the rectangular hyperbolic model predicted a slower rate of absorption and translocation of glyphosate in the GR compared with the GS biotype, though more research is needed. These results indicate possible involvement of a non-target site mechanism bestowing resistance to glyphosate. The possibility that a slow rate of glyphosate absorption and translocation might have a role in preventing the buildup of the minimum inhibitory concentration of glyphosate required at the target site needs further research.

Key words: altered absorption, *EPSPS* mutation, glyphosate metabolism, herbicide resistance, translocation.

Résumé : Lutter contre la petite herbe à poux est difficile dans le Midwest des États-Unis et l'est du Canada, car cette espèce a évolué et résiste désormais à de nombreux herbicides, dont le glyphosate. On a récemment confirmé la présence d'un biotype de petite herbe à poux 19 fois plus résistant au glyphosate, au Nebraska. La présente étude devait établir le mécanisme qui a conféré cette résistance au biotype découvert dans cet État. Les auteurs ont examiné les mécanismes de résistance avec ou sans cible en recourant à des biotypes de petite herbe à poux résistants (GR) ou sensibles (GS) au glyphosate. Les spécimens GR accumulent moins de shikimate ($\leq$ 65 μg par ml) que les spécimens GS ($\geq$ 80 μg par ml), à toutes les concentrations de glyphosate testées. Le séquençage de la zone du gène *EPSPS* conservée n'a pas révélé de mutation sur les résidus Thr_{102} ou Pro_{106}, ni aucune variation dans le nombre de copies du gène. On a cependant découvert une translocation du ^{14}C-glyphosate plus importante chez les plants GR que chez les plants GS, bien que la quantité de ^{14}C-glyphosate absorbée soit la même. Malgré cela, l'analyse des données sur l'absorption ou la translocation du ^{14}C-glyphosate au moyen du modèle hyperbolique rectangulaire prévoit un taux d'absorption et de translocation du glyphosate plus lent que celui du biotype GS dans le biotype GR, même si d'autres recherches seraient nécessaires pour le préciser. Ces résultats indiquent qu'un mécanisme sans cible pourrait intervenir dans la résistance au glyphosate. La possibilité qu'un lent taux d'absorption et de translocation du glyphosate empêche en partie l'accumulation de la quantité minimale d'herbicide nécessaire pour inhiber la plante au site ciblé mériterait qu'on entreprenne des recherches plus poussées. [Traduit par la Rédaction]

Mots-clés : modification de l'absorption, mutation du gène *EPSPS*, métabolisme du glyphosate, résistance aux herbicides, translocation.

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Z.A. Ganie and A.J. Jhala.* Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE 68583, USA.

M. Jugulam and V.K. Varanasi. Department of Agronomy, Kansas State University, Manhattan, KS 66506, USA.

Corresponding author: Amit J. Jhala (email: Amit.Jhala@unl.edu).

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Introduction

Common ragweed, a summer annual broadleaf weed, is found in diverse agroecosystems, wastelands, and roadsides (Bassett and Crompton 1975; Saint-Louis et al. 2005; Jordan et al. 2007). Common ragweed is a natural colonizer, producing 32 000 to 62 000 seeds plant^{-1} when permitted to grow for the entire season without competition from crop plants (Dickerson and Sweet 1971; Jordan et al. 2007; Friedman and Barrett 2008). Common ragweed seeds usually germinate on or near the soil surface, preferably within 5 cm depth (Stoller and Wax 1973; Jordan et al. 2007). Small seed size, specific requirements of light and temperature for germination, and a preference for undisturbed habitats has made common ragweed a predominant weed in reduced or no-till cropping systems in the midwestern United States (Jordan et al. 2007). High selection pressure due to exclusive dependence on chemical weed control in no-till cropping systems combined with a wide genetic diversity has resulted in the evolution of resistance to several herbicide sites of action in common ragweed (Schultz et al. 2000; Saint-Louis et al. 2005; Brewer and Oliver 2009; Duke and Powles 2009; Rousonelos et al. 2012). Glyphosate-resistant (GR) common ragweed was first reported in Missouri in 2004 and subsequently in 14 other states in the United States (Alabama, Arkansas, Indiana, Kansas, Kentucky, Minnesota, Mississippi, Nebraska, New Jersey, North Carolina, North Dakota, Ohio, Pennsylvania, and South Dakota) and in Ontario, Canada (Heap 2016). Additionally, common ragweed biotypes resistant to acetolactate synthase, photosystem II, and protoporphrinogen oxidase inhibitors have been reported (Patzoldt et al. 2001; Saint-Louis et al. 2005; Chandi et al. 2012; Rousonelos et al. 2012; Heap 2016).

Glyphosate is a non-selective herbicide applied after emergence with the ability to control a wide spectrum of broadleaf, grass, and perennial weeds and its lack of residual activity, low cost, and relatively safe environmental profiles (including its non-toxicity to mammals, birds, fish, or insects) has made it the most widely used herbicide throughout the world (Duke and Powles 2008; Dill et al. 2010). Glyphosate competes with phosphoenolpyruvate to irreversibly bind to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and inhibits normal function in the shikimate pathway (Funke et al. 2006; Alarcón-Reverte et al. 2013). Inhibition of the EPSPS enzyme results in unregulated carbon flow through excessive production of shikimate-3-phosphate and the insufficient synthesis of aromatic amino acids (phenylalanine, tryptophan, and tyrosine) required for protein synthesis, eventually leading to plant mortality (Schönbrunn et al. 2001; Duke and Powles 2008).

The commercialization and rapid adoption of GR crops encouraged reliance on glyphosate for broad-spectrum weed control that resulted in the evolution of GR weeds (Powles 2008; Duke and Powles 2009; Powles and Yu 2010). As of 2016, glyphosate resistance has been confirmed in 36 weed species worldwide, including 16 species in the United States (Heap 2016). Previous studies have revealed that glyphosate resistance is conferred due to one or a combination of several mechanisms, including target site mutations (Powles and Yu 2010), amplification and (or) elevated expression of the *EPSPS* gene (target site mechanisms) (Gaines et al. 2010), active vacuolar sequestration (Ge et al. 2010), limited cellular uptake, restricted translocation (Lorraine-Colwill et al. 2002), and rapid necrosis response (non-target site mechanisms) (Sammons and Gaines 2014; Van Horn and Westra 2014).

Target site mutations cause conformational changes in the structure of the EPSPS enzyme and decrease its affinity for glyphosate while maintaining the normal function of the enzyme (Funke et al. 2009). Target site mutations with the substitution of proline by serine, alanine, threonine, or leucine at position 106 (corresponding to the *Arabidopsis EPSPS* sequence) of *EPSPS* have been reported in several weed species (Sammons and Gaines 2014) and recently a double mutation with Pro_{106} to Ser and Thr_{102} to Ile substitutions conferring a high level of glyphosate resistance was reported in goosegrass [*Eleusine indica* (L.) Gaertn.] (Yu et al. 2015). Alternatively, gene amplification or elevated *EPSPS* expression leads to an increase in the level of the EPSPS enzyme, as reported first in Palmer amaranth (*Amaranthus palmeri* S Wats.), which can also confer resistance to glyphosate even though the EPSPS enzyme remains susceptible to glyphosate (Gaines et al. 2010, 2011).

In contrast, non-target site mechanisms restrict the accumulation of glyphosate at the critical and (or) toxic concentrations required to inhibit the EPSPS enzyme in the chloroplast (Powles and Yu 2010; Sammons and Gaines 2014). Non-target site mechanisms such as reduced absorption and (or) translocation of glyphosate are considered the most commonly occurring mechanisms in GR weed species (Shaner 2009; Powles and Yu 2010). In addition, several weed species with more than one mechanism of glyphosate resistance in the same population have been reported. For example, González-Torralva et al. (2012) reported impaired glyphosate translocation and glyphosate metabolism into glyoxylate, sarcosine, and aminomethylphosphonic acid as the mechanism of glyphosate resistance in a horseweed population from Spain.

Despite some earlier attempts, the precise mechanism of glyphosate resistance in common ragweed is unknown. Brewer and Oliver (2009) reported that a target site mutation and reduced absorption and translocation do not contribute to the mechanism of resistance in GR common ragweed biotypes from Arkansas. Similarly, Parrish (2015) did not find conclusive results to explain the mechanism of glyphosate resistance in a common ragweed biotype from Ohio but suggested the presence

of multiple mechanisms within the same biotype. Likewise, the mechanism of glyphosate resistance in giant ragweed (*Ambrosia trifida* L.), a closely related species to common ragweed, is also unclear, though after evaluating all possible mechanisms, Van Horn et al. (2017) ruled out the possibility of mutation at Pro_{106} or increased *EPSPS* activity and suggested that an altered translocation might be conferring the resistance. Glyphosate-resistant common ragweed confirmed for the first time in Nebraska provided an opportunity to evaluate the mechanism of glyphosate resistance in common ragweed that remains unclear based on previous studies. Therefore, the objectives of this study were to determine the mechanisms of glyphosate resistance in a common ragweed biotype from Nebraska.

Materials and Methods

Plant material and growth conditions

A common ragweed biotype from Gage County, NE (40°44′N, 96°62′W), with 19-fold glyphosate resistance (Ganie and Jhala 2017) was investigated to determine the mechanism of resistance in this study. Seeds of a known GS common ragweed biotype collected from a field near Clay Center, NE (40°52′N, 98°05′W), were used for comparison with the GR common ragweed biotype in all experiments. The plants of GR and GS common ragweed used for sample collection were treated with 1260 g a.e. ha^{-1} of glyphosate (Touchdown HiTech®, Syngenta Crop Protection, LLC, Greensboro, NC). Common ragweed seeds were germinated in plastic trays containing potting mix (Berger BM1 All-Purpose Mix, Berger Peat Moss Ltd., Saint-Modeste, QC) and after the appearance of the first true leaves, uniform-sized seedlings were transplanted to square plastic pots (8 cm × 8 cm × 9 cm) containing a 3:1 mixture of potting mix to soil. Plants were supplied with adequate water daily and fertilizers were added as 1% solution (Miracle-Gro Water Soluble All Purpose Plant Food 24-8-16, Miracle-Gro Lawn Prodcuts Inc., Marysville, OH) on a weekly basis. Uniform growth conditions were maintained for the experiments with 25 °C ± 2 °C day — 18 °C ± 3 °C night temperatures and sodium halide lamps (250 μmol m^{-2} s^{-1}) were used as a supplemental light source to ensure a 15 h photoperiod.

Shikimate assay

Common ragweed plants were grown as described in the previous section. Eight plants from each biotype were used for the shikimate assay following the protocol described by Nguyen et al. (2016). Leaf discs (5 mm in diameter) were excised from a fully expanded top leaf on each plant and placed into a single well of a 96-well flat-bottomed microtiter plate containing 0, 50, 100, 150, and 250 μmol L^{-1} glyphosate and a 10 mmol L^{-1} ammonium phosphate buffer (pH 7). The plates were incubated under fluorescent light at 560 μmol m^{-2} s^{-1} for 16 h at an incubation temperature of 26 °C–28 °C. After the incubation period, 25 μL of 0.05 mol L^{-1} HCl was added to each well and the samples were freeze-thawed through two cycles of −20 °C for 90 min followed by 60 °C for 20 min until the green color of the leaf tissues had faded away. From each well, 25 μL of the solution was transferred to fresh microtiter plates to determine shikimate levels. Shikimic acid was added to empty wells at 1, 2.5, 5, 10, 25, 50, and 100 μmol L^{-1} concentrations as standards. A mixture of 0.25% (*w*/*v*) periodic acid (H_5IO_6) and 0.25% (*w*/*v*) sodium (meta)periodate ($NaIO_4$) was added to wells of both extract and standard shikimic acid at a volume of 100 μL per well. The samples were incubated at room temperature for 60 min, after which a freshly made quench buffer (a mixture of 0.6 mol L^{-1} NaOH and 0.22 mol L^{-1} Na_2SO_3) was added (100 μL per well) to halt the reaction.

Shikimate accumulation was determined at 380 nm on a 96-well plate reader (BioTek™ Synergy™ 2 multimode microplate reader, Winooski, VT). A shikimate standard curve was developed to quantify shikimate accumulation (μg shikimate mL^{-1}) in the experimental samples (Shaner et al. 2005). The experiment was conducted in a completely randomized design with four replicates and the experiment was repeated three times. The shikimate data were subjected to analysis of variance in SAS version 9.3 (SAS Institute Inc., Cary, NC) using PROC GLIMMIX to test for treatment × experiment interaction. Shikimate accumulation data were regressed over glyphosate doses using a sigmoidal logistic regression model in the drc package of R (R statistical software, R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org):

$$(1) \qquad y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

where y is the shikimate accumulation (μg mL^{-1}) in response to glyphosate, a is the asymptote, x is the glyphosate dose (μmol L^{-1}), x_0 is the dose required to reach 50% of the maximum shikimate accumulation, and b is the slope of the curve around x_0.

EPSPS gene sequencing

Ten common ragweed plants each of the GS and GR biotypes were sampled and the experiment was repeated twice. A 100 mg sample of young leaf tissue was harvested, flash frozen, and ground into a fine powder in liquid nitrogen (−195.79 °C) using a prechilled mortar and pestle. The genomic DNA (gDNA) was extracted using DNAzol® following the manufacturer's protocol (Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA). Quality and concentration of gDNA were determined by using gel electrophoresis (0.8% agarose) and a NanoDrop™ (ND-1000) spectrophotometer (Thermo Fisher). A polymerase chain reaction (PCR) was performed on gDNA in a T100 thermal cycler (BioRad Inc.,

Table 1. Primers used for sequencing conserved region of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) and for real-time quantitative PCR (qPCR) in glyphosate-resistant and -susceptible common ragweed biotypes from Nebraska.

Gene	Primer sequence	Amplicon size (bp)	T_m (°C)	Reference
EPSPS (Thr_{102}, Pro_{106})	F: 5′-ATGTTGGACGCTCTCAGAACT-3′ R: 5′-TGAATTTCCTCCAGCAACGGC-3′	195	56	Wiersma et al. 2015
EPSPS (qPCR)	F: 5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′ R: 5′-TGAATTTCCTCCAGCAACGGCAA-3′	195	59	Gaines et al. 2010
EPSPS (qPCR)	F: 5′-AGGGTTGTGGTGGTCTGTTTCC-3′ R: 5′-ATTTTCCTCCAGCAACGGCAAC-3′	123	59	(Z.A. Ganie et al., unpublished data)
β-tubulin	F: 5′-ATGTGGGATGCCAAGAACATGATGTG-3′ R: 5′-TCCACTCCACAAAGTAGGAAGAGTTCT-3′	157	59	Godar et al. 2015

Note: T_m, melting temperature.

Hercules, CA) to amplify the conserved region of *EPSPS* covering Pro_{106} and Thr_{102} codons with the primers used by Wiersma et al. (2015) (Table 1). Each 50 μL reaction volume consisted of 25 μL of PCR master mix, 5 μL of forward primer (5 μmol L^{-1}), 5 μL of reverse primer (5 μmol L^{-1}), 3 μL of gDNA template (15 ng μL^{-1}), and 12 μL of nuclease-free water. The thermocycler conditions for PCR were initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 56 °C for 30 s, product extension at 72 °C for 1 min, and a final extension cycle at 72 °C for 5 min. The PCR products were run on 1% agarose gel stained with ethidium bromide using 500 and 100 bp markers to confirm amplicon size (195 bp). Polymerase chain reaction products were purified using a GeneJet PCR purification kit (Thermo Fisher) and quantified using a NanoDrop™ spectrophotometer. About 15 μL of the purified PCR product (25 ng μL^{-1}) was sequenced at the Kansas State University sequencing facility using an ABI 3730 DNA analyzer (Applied Biosystems, Thermo Fisher). MultAlin software was utilized to align and analyze the *EPSPS* nucleotide sequences for the presence of any known target site mutation(s) reported to confer glyphosate resistance (Corpet 1988).

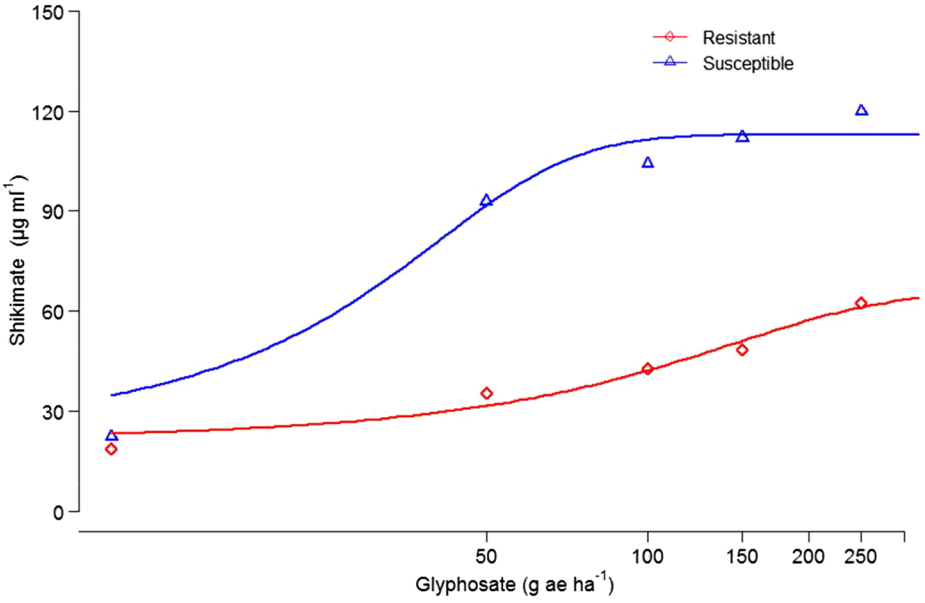
Relative *EPSPS* genomic copy number

The genomic DNA of eight GR and four GS plants was used for real-time quantitative PCR (qPCR) to determine the *EPSPS* gene copy number using *β-tubulin* as a reference gene for normalization. The *EPSPS* gene copies were measured relative to the calibrator sample (a known GS biotype). The qPCR was performed using a StepOnePlus™ Real-Time PCR System (Thermo Fisher) and the primer sequences used in the qPCR are presented in Table 1. Additional common ragweed specific qPCR primers were designed based on the *EPSPS* sequence obtained in this study and used to confirm the results obtained with the previous set of primers (Table 1). The common ragweed specific qPCR primers were designed using OligoAnalyzer 3.1 (IDT SciTools, 2014; Integrated DNA Technologies, Inc., Coralville, IA). The reaction mix for qPCR consisted of 8 μL of SYBR Green mastermix (BioRad), 2 μL each of the forward and reverse primers (5 μmol L^{-1}), and 2 μL of gDNA (15 ng μL^{-1}) to bring the total reaction volume to 14 μL. The qPCR thermal specifications were 95 °C for 15 min, 40 cycles of 95 °C for 30 s, and 60 °C for 1 min, followed by a melt curve analysis. The melt curve profile was generated to determine the specificity of the qPCR reaction and the amplification efficiency was always equal to 1. The relative gene copy number was determined by using the $2^{-\Delta\Delta CT}$ method, where CT is the threshold cycle and ΔΔCT is $CT_{Targetgene\ (EPSPS)} - CT_{Referencegene\ (\beta\text{-}tubulin)}$ (Gaines et al. 2010).

Absorption and translocation of glyphosate

Seeds of common ragweed biotypes were germinated in plastic trays containing potting mix (Berger BM1 All-Purpose Mix, Berger Peat Moss Ltd.) and uniform-sized plants were transplanted at the two-leaf stage and shifted to a growth chamber at 4 d after transplanting. The plants were maintained at 28 °C ± 2 °C day — 22 °C ± 2 °C night temperatures, 75% (±4%) relative humidity, and a 15 h photoperiod. Eight to ten centimetre tall plants were selected for absorption and translocation experiments and sprayed with 1260 g a.e. ha^{-1} of glyphosate after covering a fully expanded young leaf with plastic wrap (Saran™ Premium Wrap, Racine, WI). The plastic wrap was carefully removed after the spray and the leaf was marked. Within 1 h of glyphosate spray, ten 1 μL droplets of ^{14}C-glyphosate (0.33 kBq μL^{-1}) (PerkinElmer Inc., Boston, MA) were applied to the upper surface of the marked leaf using a microapplicator. The ^{14}C-glyphosate solution was prepared by mixing ^{14}C-glyphosate with a commercial formulation of glyphosate (Touchdown HiTech®, Syngenta) and distilled water to achieve a final concentration equivalent to 1260 g a.e. ha^{-1}. Plants were dissected at 8, 24, 48, 72, 96, 120, and 168 h after treatment (HAT) into treated leaf (TL), tissues above treated leaf (ATL), tissues below treated leaf (BTL), and roots. The treated leaf was cut at the point of attachment to the stem and the roots were washed over wire mesh to

Fig. 1. Accumulation of shikimate in leaf discs of the glyphosate-resistant and -susceptible common ragweed biotypes at 24 h after treatment with increasing glyphosate concentrations. Each data point represents the mean amount of shikimate accumulation pooled from three experiments each with three replicates at each glyphosate concentration. [Colour online.]



remove soil. Treated leaves were rinsed twice in a 20 mL scintillation vial containing 5 mL wash solution (1:1 *v*/*v* mixture of methanol and deionized water and 0.05% Tween® 20 (Sigma-Aldrich Corp., St. Louis, MO) for 1 min to remove the unabsorbed herbicide from the surface of the treated leaf. The leaf rinse was mixed with 15 mL of scintillation cocktail and the radioactivity was determined by using liquid scintillation spectrometry (LSS) (Tricarb 2100 TR Liquid Scintillation Analyzer; Packard Instrument Co., Meriden, CT). Plant sections were dried at 55 °C for 72 h and combusted in a biological oxidizer (OX-501, RJ Harvey Instruments, NY) to recover ^{14}C-labelled glyphosate in a proprietary ^{14}C-trapping scintillation cocktail and radio-assayed using LSS. Herbicide absorption and translocation were calculated as per Godar et al. (2015):

$$(2)\quad \%\ \text{absorption} = \left(\frac{\text{total radioactivity applied} - \text{radioactivity recovered in wash solution}}{\text{total radioactivity applied}} \times 100\right)$$

$$(3)\quad \%\ \text{translocation} = 100 - \%\ \text{radioactivity in treated leaf}$$

where % radioactivity in treated leaf = (radioactivity recovered in treated leaf/radioactivity absorbed) × 100.

The experiments were arranged in a randomized complete block design by blocking to overcome variability due to plant size with four replications and the experiment was repeated twice. Data from absorption and translocation experiments were subjected to analysis of variance in SAS version 9.3 (SAS Institute Inc.) using PROC GLIMMIX. Common ragweed biotypes (GR and GS), harvest time, and their interactions were considered fixed effects and the experimental runs were considered as random effects. However, significant biotype × time interaction for absorption and translocation warranted further exploration of the data using the regression analysis to include the time structure of the observations (Grangeot et al. 2006; Burke et al. 2007; Kniss et al. 2011; Nandula and Vencill 2015). A rectangular hyperbolic model was selected from the models reported in the literature based on Akaike's information criterion to explain the relationship of the measured responses over time (Burke et al. 2007; Kniss et al. 2011). The rectangular hyperbolic model was fit to the data using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA):

$$(4)\quad y = \frac{(A_{\max} \times t)}{(1 \times t_{50} + t)}$$

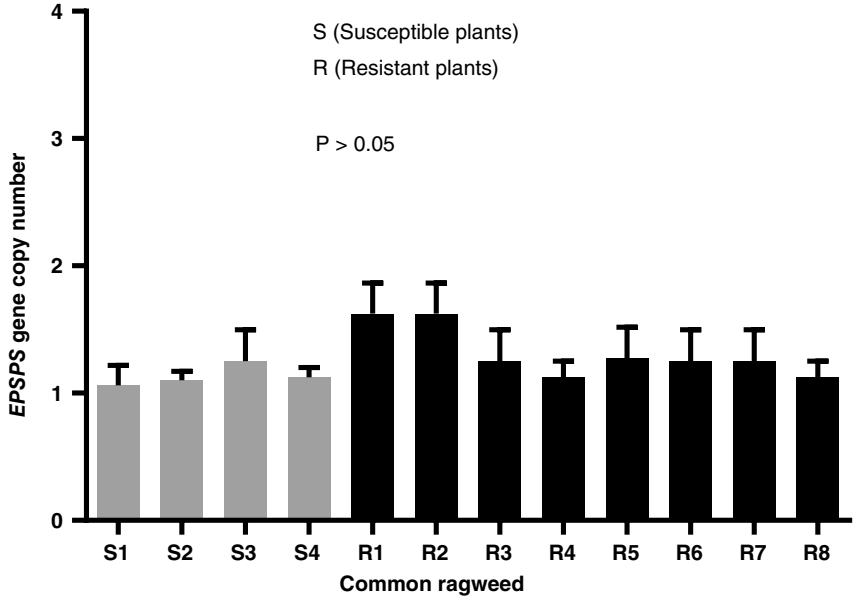
$$(5)\quad y = \frac{(A_{\max} \times t)}{(0.11 \times t_{90} + t)}$$

Table 2. Nucleotide bases and predicted amino acid sequence of the conserved region of the *EPSPS* gene covering Thr_{102} and Pro_{106} from glyphosate-resistant (GR) and -susceptible (GS) common ragweed biotypes from Nebraska.

Amino acid no.	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
Amino acid name	Leu	Gly	Asp	Ala	Gly	Thr	Ala	Met	Arg	Pro	Leu	Thr	Ala	Ala	Val
Consensus sequence	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
Reference 1 (Palmer amaranth) accession no. FJ861243.1	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
Reference 2 (Spiny amaranth) accession no. KF569211.1	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR1	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR2	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR3	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR4	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR5	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR6	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR7	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GR8	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GS1	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GS2	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GS3	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GS4	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GS5	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GS6	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT
GS7	CTT	GGT	AAT	GCA	GGA	**ACA**	GCG	ATG	CGC	**CCA**	TTG	ACA	GCT	GCG	GTT

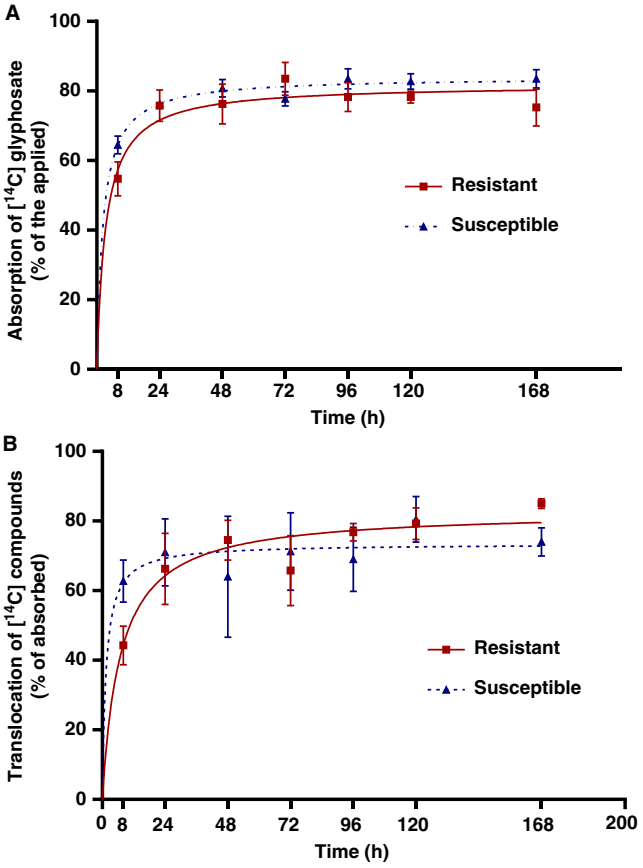
Note: Bold values indicate location of Thr_{102} or Pro_{106} residues where mutation was previously reported in some glyphosate-resistant species.

Fig. 2. 5-Enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene copy number in glyphosate-susceptible (GS) and -resistant (GR) biotypes from Nebraska. *EPSPS* gene copy number was measured relative to a calibrator sample (S1). Error bars represent ± standard error from the mean ($n = 3$ replicates). The real-time quantitative PCR data were normalized using *β-tubulin* as a reference gene. The *EPSPS* gene copy number did not vary between the GR and GS biotypes ($P > 0.05$).



where y is the percentage of the applied ^{14}C-glyphosate absorbed or translocated in the plant, A_{max} is the asymptote or maximum absorption or translocation expressed as the percent applied, t is the time (h) after herbicide application, and t_{50} or t_{90} is the time required for 50% or 90% of the maximum absorption or translocation to occur.

Fig. 3. A pattern of ^{14}C-glyphosate (A) absorption and (B) translocation in glyphosate-resistant and -susceptible common ragweed biotypes from Nebraska. Each data point represents the means based on two experiments each with four replicates. Vertical bars are the standard error of mean. [Colour online.]



Metabolism of glyphosate

Glyphosate-resistant and GS common ragweed plants (6–8 cm tall) were selected and treated with ^{14}C-glyphosate as described above in the absorption and translocation study, the only difference being that fifteen 1 μL droplets of ^{14}C-glyphosate (0.33 kBq μL^{-1}) were applied to facilitate the recovery and easy detection of radioactivity. At 48 and 96 HAT, the treated leaves were harvested and rinsed as described in the absorption and translocation study. Whole plant tissues including the washed treated leaf were then frozen in liquid nitrogen and homogenized with a prechilled mortar and pestle. ^{14}C-glyphosate and its metabolites were extracted with 15 mL of 25% acetonitrile at 20 °C for 30 min and samples were centrifuged at 6500 rev min^{-1} (5000*g*) for 25 min. Supernatant was concentrated at 50 °C for 2–4 h depending on the rate of evaporation until a final volume of 600 μL was reached with a rotary evaporator (Centrivap, Labconco, Kansas City, MO). About 600 μL of the extract was transferred to a 1.5 mL micro centrifuge tube and centrifuged at a high speed (13 000 rev min^{-1}; 10 000*g*) for 20 min. Radioactivity in each sample was measured by LSS before high-performance liquid chromatography (HPLC) analysis and the samples were normalized to 60 dpm μL^{-1} (amount of ^{14}C compounds) by diluting the samples with 25% acetonitrile (Godar et al. 2015).

Total extractable radioactivity in 50 μL of the samples was resolved into parent glyphosate and its polar metabolites by reverse-phase HPLC (System Gold, Beckman Coulter, Pasadena, CA). Reverse-phase HPLC was performed with a Zorbax SAX Column (4.6 mm × 250 mm, 5 μm particle size; Agilent Technologies, Santa Clara, CA) at a flow rate of 1 mL min^{-1} with eluent A (1–5 mmol L^{-1} KH_2PO_4, pH = 2) and eluent B (1–100 mmol L^{-1} KH_2PO_4, pH = 2) (Pollard et al. 2004). The elution profile was programmed as 0% B for 1 min and 0%–100% B in 12 min. In between injections, solvent B was used to wash and solvent A was used to re-equilibrate the columns. The retention time of the parent compound, ^{14}C-glyphosate, was determined by injecting 50 μL of 60 dpm μL^{-1} ^{14}C-glyphosate diluted with 25% acetonitrile. The parent compound was detected by a radio flow detector and displayed a retention time of 12.65 min. The treatments were replicated four times and the experiment was repeated twice.

Results and Discussion

Shikimate accumulation

Treatment × experiment interaction for shikimate accumulation was not significant; therefore, data were combined over three experiments. Both GR and GS common ragweed biotypes showed shikimate accumulation in response to glyphosate; however, higher shikimate accumulation was observed in the GS biotype at all glyphosate concentrations (Fig. 1). The estimated parameters of the logistic regression model for shikimate accumulation to glyphosate concentration were $y = 65/\{1 + (x/56)^{-0.013}\}$ with a root mean square error of 5.4 for the GR biotype and $y = 113/\{1 + (x/24)^{-0.057}\}$ with a root mean square error of 4.6 for the GS biotype, where y represents the shikimate accumulation (μg mL^{-1}) and x represents the glyphosate concentration (μmol L^{-1}). The model predicted a maximum shikimate accumulation of 113 μg mL^{-1} in the GS biotype compared with 65 μg mL^{-1} in the GR biotype. The model also predicted that the glyphosate concentration required to reach 50% of the maximum shikimate accumulation in the GS biotype was 24 μmol L^{-1}, compared with 56 μmol L^{-1} in the GR biotype. Results indicated that the EPSPS enzyme in the GS biotype had 2.3 times greater sensitivity to glyphosate compared with the GR biotype. Similarly, Pollard et al. (2004) reported 3-fold more shikimate accumulation in GS common ragweed from Missouri compared with a GR biotype. Norsworthy et al. (2010) also reported 3.3- to 3.8-fold more shikimate accumulation in GS giant ragweed compared with the GR biotype. In contrast,

Table 3. Regression parameters for the absorption and translocation of ^{14}C-glyphosate in GR and GS common ragweed biotypes from Nebraska.[a,b]

Movement of ^{14}C-glyphosate	Common ragweed biotype	Regression parameters[c]		
		A_{max}	t_{50}	t_{90}
Absorption into treated leaf	GS	84 (1.0)	2.4 (0.2)	22 (2.0)
	GR	82 (1.5)	3.4 (0.6)	31 (5.5)
P value		0.070	0.013	0.016
Total translocation into plant	GS	73 (2.4)	1.5 (0.9)	26 (8.0)
	GR	84 (3.0)	7.6 (1.4)	69 (13.0)
P value		0.015	0.012	0.011
Translocation to tissues above the treated leaf	GS	14 (0.6)	4.4 (1.5)	27 (12.0)
	GR	13 (1.0)	11.1 (4.7)	64 (7.0)
P value		0.102	0.023	0.033
Translocation to aboveground tissues below the treated leaf	GS	6 (0.4)	1.8 (0.5)	3 (1.0)
	GR	5 (0.4)	4.5 (1.3)	6 (1.0)
P value		0.050	0.011	0.035
Translocation to roots	GS	15 (1.4)	9.0 (3.6)	9 (5.0)
	GR	17 (2)	7.1 (2.4)	12 (6.0)
P value		0.131	0.451	0.407

[a]Parameter estimates for the rectangular hyperbolic model fit to the absorption and translocation data $y = (A_{max} \times t)/(0.11 \times t_{90} + t)$ where y is the percentage of the applied ^{14}C-glyphosate absorbed or translocated in the plant, A_{max} is the asymptote or maximum absorption or translocation expressed as the percent applied, t is the time (h) after herbicide application, and t_{90} is the time required for 90% of the maximum absorption or translocation to occur.

[b]The predicted parameters of the GR and GS biotype were compared using the t test and the P values are presented.

[c]Values in parentheses are standard errors.

Brewer and Oliver (2009) reported an identical pattern of shikimate accumulation in GR and GS biotypes of common ragweed from Arkansas, though shikimate accumulation stabilized in the GR biotype at 3 DAT but continued to increase in the GS biotype.

The accumulation of shikimate in the GR biotype of common ragweed provided evidence about the sensitivity of *EPSPS* to glyphosate and suggested that glyphosate was able to enter and accumulate in the cells and the chloroplasts. A rise in shikimate levels with increasing glyphosate concentrations possibly occurred due to more glyphosate buildup at the target site, leading to an increase in shikimate accumulation. Earlier, based on the results of the shikimate assays, non-target site mechanisms have been suggested for glyphosate resistance in horseweed (Koger and Reddy 2005; Nol et al. 2012) and giant ragweed (Norsworthy et al. 2010).

Target site mutation

The region of *EPSPS* about 145 bp long covering the Thr_{102} and Pro_{106} residues was sequenced to identify the point mutations ($Pro_{106}Ser$ and $Thr_{102}Ile$) known to confer glyphosate resistance. There were no differences in the *EPSPS* sequence of the GR and GS common ragweed biotypes (Table 2). These results suggest that glyphosate resistance in common ragweed from Nebraska did not evolve as a result of mutations in the *EPSPS* gene. Similarly, Nandula et al. (2015) reported that amino acid substitution at codon 106 in *EPSPS* was not present in GR common ragweed from Mississippi.

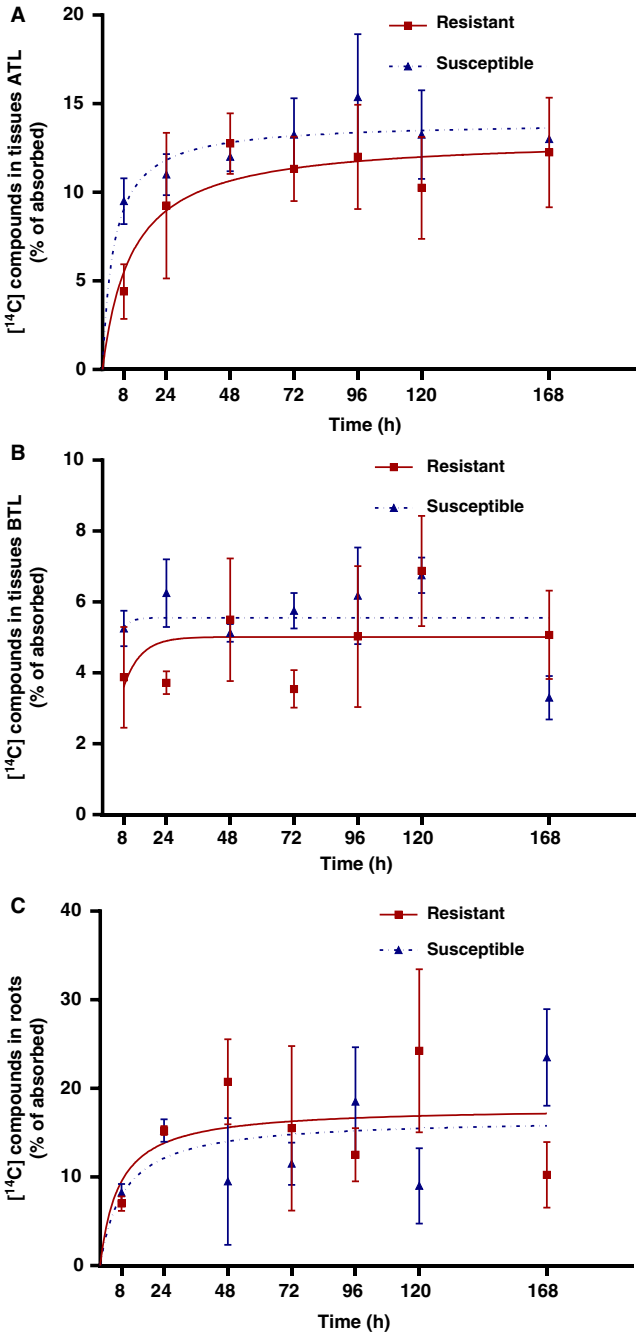
Relative *EPSPS* genomic copy number

The qPCR results exhibited no differences in the *EPSPS* gene copy number between the GR and the GS biotypes. The relative *EPSPS* gene copy number varied from 1 to 2 (Fig. 2) and no amplification of *EPSPS* was observed in the GR biotype to explain the basis of glyphosate resistance. Similar to the results of this study, *EPSPS* gene amplification was not the mechanism of glyphosate resistance in giant ragweed biotypes from across the United States and Canada (Van Horn et al. 2017).

Absorption and translocation of glyphosate

Treatment × experiment interaction for glyphosate absorption and translocation was not significant; therefore, data were pooled over the two experiments. Recovery of ^{14}C-glyphosate was similar in GR and GS biotypes across the experiments. More than 80% of ^{14}C-glyphosate applied was recovered at 8 HAT, followed by 69%–70% at 24, 48, 72, and 96 HAT and 60%–65% at 168 HAT. A similar pattern of ^{14}C-glyphosate recovery was

Fig. 4. Percentage of ^{14}C-glyphosate translocated to plant sections including (A) tissues above the treated leaf (ATL), (B) aboveground tissues below the treated leaf (BTL), and (C) roots at different harvest time points (8, 24, 48, 72, 96, 120, and 168 h after treatment) after the application of ^{14}C-glyphosate to glyphosate-resistant and -susceptible common ragweed biotypes from Nebraska. Each data point represents the mean based on two experiments each with four replicates. Vertical bars are the standard error of mean. [Colour online.]



reported in a common ragweed biotype from Arkansas, with ≥80% recovery at 6 HAT and 68%–79% recovery at 48 HAT (Brewer and Oliver 2009).

Total absorption expressed as the percent of applied ^{14}C-glyphosate was similar in GR (82%) and GS (84%) biotypes (Fig. 3A). Brewer and Oliver (2009) reported that mean absorption varied from 38% to 80% of the applied ^{14}C-glyphosate at 24 HAT in common ragweed biotypes from Arkansas without any differences between the GR and GS biotypes. Similarly, Nandula et al. (2015) reported a same pattern of glyphosate absorption in the GR and GS biotypes of giant ragweed with 17–18 h required to complete 50% of absorption. However, in this study, the rectangular hyperbolic model predicted a rapid absorption of glyphosate in the GS common ragweed biotype compared with the GR biotype. The time required for 50% and 90% absorption of ^{14}C-glyphosate to occur in the GS plants was 2.4 and 22 HAT compared with 3.4 and 31 HAT in the GR plants (Table 3). In contrast, Grangeot et al. (2006) reported 100% uptake of ^{14}C-glyphosate in a common ragweed biotype at 24 HAT with 50% absorption completed within 3 HAT.

Interaction between the biotypes and time of harvest of plant samples was significant with respect to the translocation of ^{14}C-glyphosate (data not shown). The results indicated 73% and 84% translocation of the absorbed ^{14}C-glyphosate in the GS and GR biotypes, respectively (Fig. 3B; Table 3). Reduced translocation in susceptible plants possibly occurred due to the effect of glyphosate on the photosynthesis and carbon export processes in the source leaves, along with glyphosate-induced inhibition of the assimilate metabolism in sink tissues (Geiger and Bestman 1990; Geiger et al. 1999). Similarly, Nandula et al. (2015) reported greater ^{14}C-glyphosate translocation in the GR compared with the GS common ragweed biotype. However, the GS biotype showed a rapid rate of translocation, with 50% and 90% of the total translocation completed within 1.5 and 26 HAT, respectively, compared with 7.6 and 69 HAT required for the GR biotype (Table 3). Geiger et al. (1999) observed that export of glyphosate ceased by 10 HAT in susceptible sugar beet (*Beta vulgaris*) plants, while it continued in the GR plants up to a period of 30 HAT. Similarly, translocation continued for 2–3 d after treatment (DAT) in conventional corn (*Zea mays* L.) compared with 5 DAT in GR corn (Hetherington et al. 1999). ^{14}C-glyphosate translocated to tissues above the treated leaf varied from 13% to 14% of the absorbed quantity and did not differ between the two biotypes (Fig. 4A; Table 3). Similarly, ^{14}C-glyphosate translocated to aboveground tissues below the treated leaf and to the roots did not differ between GR and GS biotypes (Figs. 4B and 4C). Though, the regression parameters suggest more time was required to complete 50% and 90% translocation to different plant sections, including tissues above or below the treated leaf in GR compared with GS common ragweed (Figs. 4A, 4B; and Table 3); however, it did not explain the precise mechanism involved, but indicated that additional evidence related to the leaf or phloem loading and subcellular

distribution of glyphosate is needed to reach a conclusion about the precise mechanism. Feng et al. (1999) reported delayed and decreased leaf loading and export of glyphosate in the treated leaf of GR horseweed compared with the GS treated leaf. Similarly, Nandula et al. (2015) reported a higher rate of translocation in the GS giant ragweed biotype compared with the GR biotype. Additionally, the nonlinear regression parameters indicated that 50% translocation occurred within 21.8 HAT in the GR biotype compared with 9.9 HAT in the GS biotype and results were confirmed by phosphor imaging (Nandula et al. 2015).

Glyphosate metabolism

The results of reverse-phase HPLC demonstrated that no metabolism of glyphosate occurred in either the GR or GS biotypes at 48 or 96 HAT (data not shown). These results indicated that metabolic deactivation or decomposition does not contribute to glyphosate resistance in common ragweed from Nebraska.

The results from this study indicated that target site mechanisms including previously known point mutations (Pro_{106} to Ser and Thr_{102} to Ile) or amplification of the *EPSPS* gene did not contribute to the mechanism of glyphosate resistance in a common ragweed biotype from Nebraska. These results are in consensus with shikimate accumulation, suggesting that the EPSPS enzyme in the GR biotype was inhibited by glyphosate, though the level of sensitivity was reduced compared with the GS biotype (Fig. 1). The shikimate accumulation in the GR biotype may also be interpreted as the presence of sensitive EPSPS in addition to altered EPSPS enzyme with a different mutation than previously known mutations; however, this potential hypothesis needs more research to confirm it. Absorption and translocation experiments revealed that total glyphosate absorption was similar in both common ragweed biotypes but a more rapid rate of absorption was observed in the GS biotype compared with the GR biotype (Fig. 3A). In contrast, overall translocation was slightly higher in the GR biotype (Fig. 3B); however, the time required to complete 90% of the translocation was 2.6 times greater in the GR biotype compared with the GS biotype (Table 3). The slow rate of absorption and translocation in the GR biotype might be due to reduced loading and movement of glyphosate caused by the alterations in the transporters involved in glyphosate transport within the plant system. The results of this study do not provide sufficient evidence to explain the precise mechanism of glyphosate resistance in common ragweed; however, they certainly provided a new direction for future research needed.

The molecular mechanisms resulting in slow absorption and translocation of glyphosate in GR biotype is not clear; however, the possibilities may include the presence of barriers interfering with glyphosate loading into the phloem or within cell movement and subcellular distribution. Earlier studies have categorized common ragweed as a species with little glyphosate uptake (Sammons and Gaines 2014) and it was speculated that the plasma membrane transporters mediate the glyphosate exclusion from the plant cells in GR common ragweed (Ge et al. 2013). Several processes of non-target site mechanisms of glyphosate resistance reported earlier in other weed species of the Asteraceae family should be helpful to further investigate the mechanism of glyphosate resistance in common ragweed. For example, the role of adenosine triphosphate binding cassette (ABC) transporters in the sequestration of glyphosate into vacuoles (Ge et al. 2010) or the upregulation of several *ABC transporter* genes has been reported in GR horseweed (Peng et al. 2010; Nol et al. 2012). Additionally, a recent study in GR hairy fleabane reported that glyphosate was not able to reach the target enzyme despite its presence in the cells due to impaired subcellular distribution that resulted in glyphosate inaction (Kleinman and Rubin 2017).

In conclusion, a non-target site based resistance mechanism suspected as an insufficient amount of glyphosate at the target enzyme due to slow rates of absorption and translocation may have contributed to resistance in a common ragweed biotype from Nebraska. However, further research is needed to examine the differences in subcellular distribution of glyphosate and tonoplast membrane transporters between GR and GS common ragweed biotypes.

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