Research Article

Received: 11 January 2018 Revised: 16 April 2018 Accepted article published: 2 May 2018 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.5061

Multiple target site resistance to glyphosate in junglerice (*Echinochloa colona*) lines from California orchards

Sarah Morran,[a] Marcelo L Moretti,[b] Caio A Brunharo,[a] Albert J Fischer[a] and Bradley D Hanson[a]*

ABSTRACT

BACKGROUND: In California specialty cropping systems such as vineyards and orchards, *Echinochloa colona* is present as a summer annual weed. It is able to germinate throughout the growing season whenever favorable conditions are present, and management relies heavily on glyphosate applications. Glyphosate-resistant (GR) *E. colona* biotypes are present in the state, but the levels of resistance observed suggest that there may be differences in mechanisms of resistance among populations.

RESULTS: *Echinochloa colona* lines collected from different regions of California's Central Valley presented resistance levels ranging from 1.4 to 4.3-fold compared to susceptible lines. No differences in the absorption and translocation of [^{14}C]-glyphosate were observed among lines. Resistant lines accumulated eight-fold less shikimic acid after treatment with 435 and 870 g a.e. ha^{-1} glyphosate compared to the most susceptible line. Sequencing of a region of the *EPSPS* gene revealed three single nucleotide changes leading to amino acid substitutions at Proline 106, including Pro106Leu, Pro106Thr and Pro106Ser.

CONCLUSION: These results indicate that an altered target site in EPSPS is contributing to resistance in these lines and resistance has evolved independently, multiple times in the Central Valley of California. Additional research is needed to further understand the genomic contributions of resistance loci in this polyploid weed species.

Keywords: *Echinochloa colona*; *EPSPS*; 5-enolpyruvylshikimate-3-phosphate synthase; glyphosate; herbicide resistance

1 INTRODUCTION

Echinochloa colona (L.) Link is a tropical C_4 annual grass weed present in the major agricultural systems of over 60 countries and rated among the world's worst weeds.[1] *Echinochloa colona* is becoming a primary weed of tree nut and vineyard cropping systems in California, a cropping system that aggregates as a $25.3 billion dollar industry[2] occupying over 925 000 ha in the state.[3] Tree nut and vineyard crops are heavily reliant on postemergence glyphosate for weed control; thus the presence of glyphosate-resistant (GR) *E. colona*, and other GR weeds, makes weed management a challenge in these systems. Glyphosate resistance in *E. colona* was first reported in Australia[4] and later in Argentina and Venezuela.[5] In 2013, the first case of GR *E. colona* in the United States was identified and characterized from a *Zea mays* field in northern California.[6] Resistance in this *E. colona* population was attributed to a target site mutation of Pro106Ser.

Target site resistance can refer to mutations in the *EPSPS* gene that affect the interaction of glyphosate with EPSPS, gene duplication of *EPSPS* in the plant genome or altered expression of *EPSPS*. A number of mutations in the *EPSPS* gene have been associated with glyphosate resistance. The most commonly reported point mutations are at amino acid 106 resulting in substitutions from proline to alanine, leucine, serine or threonine. An amino acid change at position 106 causes a change in the structure of the active site reducing the space for glyphosate to bind while maintaining affinity to phosphoenolpyruvate (PEP).[7,8] In addition to single point mutations, double mutations have been associated with high levels of glyphosate resistance in transgenic *Zea mays* (Gly101Ala + Gly144Asp)[9] and *Brassica napus* (Gly101Ala + Ala192Thr)[10] and the weed species *Eleusine indica* (Thr102Ile + Pro106Ser).[11] Duplication of *EPSPS* was first reported in the weed *Amaranthus palmeri*[12] and since then has been reported in other weed species. Most reports have shown that mRNA and EPSPS protein levels have a linear relationship with copy number and reduction in plant growth.[13] Resistance levels conferred through target site mechanisms can range from low to high depending on the species. A Pro106Thr substitution in *Lolium rigidum* is associated with a two- to three-fold increase in resistance to glyphosate[8] whereas Pro106Ala mutation in a *L. multiflorum* population showed 5–15 fold resistance.[14]

Resistance to glyphosate in weeds can also be conferred by non-target site mechanisms, such as altered translocation,

* *Correspondence to: BD Hanson, Department of Plant Sciences, University of California, One Shields Ave., Davis, CA 95616, USA. E-mail: bhanson@ucdavis.edu*

a *Department of Plant Sciences, University of California, Davis, CA, USA*

b *Department of Horticulture, Oregon State University, Corvallis, OR, USA*

reduced uptake and vacuolar sequestration, which are reviewed in depth by Sammons and Gaines.[13]

The altered translocation of glyphosate via non-target site mechanisms can enable high levels of resistance in many plant species. Studies have identified both reduced absorption and reduction in the movement of glyphosate out of the treated leaf as processes contributing to resistance. In the perennial grass *Chloris elata* Desv., resistant plants absorbed less glyphosate and retained more glyphosate in the treated leaf compared to susceptible lines.[15] More specifically, the sequestration of glyphosate into the vacuole via active transport has been determined as one cellular process leading to reduced translocation in some resistant plants. Ge *et al.*[16] demonstrated that in resistant *L. rigidum* and *L. multiflorum* sequestration into the vacuole is via an active transporter in the tonoplast, and that the sequestration was unilateral.[16] Studies of GR *Conyza canadensis* gene expression identified genes from the large ABC-transporter family that may be involved in this process.[17] Recent evidence suggests exclusion of glyphosate from the cytoplasm and restricted entry of glyphosate into the chloroplast are cellular processes that can also contribute to resistance via altered translocation.[18]

Initial work in *E. colona* from California documented the presence of different glyphosate resistance mechanisms in populations tested from the northern Central Valley.[6] Because of increasing reports from growers of unsatisfactory control of *E. colona* with glyphosate in perennial cropping systems in the state (B.D. Hanson, personal communication), a subsequent survey was conducted on *E. colona* collected from orchards across the Central Valley in 2010–2011.[19] Glasshouse screening identified multiple populations with varying levels of resistance to glyphosate and target site mutations at Pro106 were detected in these resistant populations.[19] The varying levels of resistance observed in the field-collected populations raised questions as to how different mechanisms, including several different Pro106 mutations, may contribute to the level of resistance in polyploid weeds. Here, we further investigate the mechanisms of glyphosate resistance in *E. colona* using F_4 single seed selfed lines developed from the original 2010–2011 field collections to minimize genetic variability in this polyploid species.

2 MATERIALS AND METHODS

2.1 Plant material and phenotype characterization

Echinochloa colona seed was collected from 35 orchard sites in the Central Valley of California.[19] Five previously uncharacterized lines from this survey consisted of two susceptible (A3, C6) and three resistant (A8, N3, SV2) which were selected for further analysis based on their varying resistance levels and morphology. Two resistant lines, H1 and H5, that were initially characterized as field-collected populations,[19] were also included to contrast and characterize results when the variability in genetic background had been reduced. The least visibly-injured survivors of 435 or 870 g a.e. ha^{-1} from each field population were subsequently developed into single seed selfed F_4 lines with selection at each generation. For all experiments on the F_4 lines, seeds were chemically scarified for 30 min in concentrated (95–99%) sulfuric acid followed by rinsing in deionized water.[20] Seeds were treated with a 0.2% *w/v* captan solution and germinated at room temperature on moist blotter paper. After germination, seedlings were transplanted into 7.5 cm × 7.5 cm × 10 cm pots filled with commercial potting media (Sun Gro Horticulture Canada Ltd, Vancouver, BC, Canada). Plants were glasshouse grown at 25–30 °C and 16 h day length. Natural light was supplemented by metal halide lamps at 900 $\mu mol^{-2}\ s^{-1}$ photosynthetic photon flux density. Plants were treated at the 3–4 leaf stage with glyphosate (Roundup Powermax, 540 g a.e. L^{-1}, Monsanto, St Louis, MO, USA) at 0, 108.75, 217.5, 435, 870, 1740, 3480, 5220, 6960 and 8700 g a.e. ha^{-1} in a spray chamber using an 8002E flat-fan nozzle (TeeJet Technologies, Wheaton, IL, USA) delivering 120 L ha^{-1} at 250 kPa. Nine seedlings were grown per pot with three replicates per glyphosate rate. Twenty-one days after treatment, mortality and dried aboveground biomass was recorded for each individual plant. This experiment was conducted twice.

2.2 Non-target-site resistance

To explore the involvement of non-target site mechanisms, plants were evaluated for glyphosate absorption and translocation, and glyphosate metabolism.

2.2.1 *Absorption and translocation of [^{14}C] glyphosate*

The absorption and translocation of glyphosate in *E. colona* lines was determined as described by Moretti and Hanson,[21] with minor modifications. In brief, plants were grown in 2.5 cm × 2.5 cm polypropylene pots containing commercial potting mix (Sun Gro Horticulture Canada Ltd). At the 2–3 leaf stage, soil was washed from roots and plants were transferred to glass vials filled with Hoagland's nutrient mixture (40 mL) (MP Biomedical, Santa Ana, CA, USA). At the 3–4 leaf stage, three droplets (1 μL) containing a total 1.4 kBq of [^{14}C]-glyphosate [phosphonomethyl-^{14}C] (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) were applied to the adaxial surface of the youngest fully expanded leaf. The treatment solution contained [^{14}C]-glyphosate (specific activity of 2.22 GBq/mmol) plus Roundup Powermax at 15 mM to approximate a 500 g a.e. ha^{-1} in 200 L ha^{-1} application. Treated plants were maintained at a 16 h photoperiod and 24/18 °C day/night temperatures. Plants were destructively harvested at 6, 24 and 48 h after treatment (HAT) and sectioned into treated leaf, all other above ground tissue, and root tissue. Tissue samples were dried at 50 °C for 72 h and subsequently combusted in a biological oxidizer (307, PerkinElmer, Waltham, MA, USA). Hence, $^{14}CO_2$ was trapped in a (20 mL) cocktail of Carbo-Sorb E® (PerkinElmer) (10 mL) and Permafluor® (PerkinElmer) (10 mL) and quantified by liquid scintillation spectrometry (Tricarb 4810TR, PerkinElmer). Each harvest time point had five biological replicates and the study was conducted twice.

2.2.2 *Glyphosate metabolism*

Lines were grown and transferred to hydroponic solutions as described earlier. Ten 1-μL droplets of [^{14}C]-glyphosate were placed on the youngest fully expanded leaves when plants were at the 2–3 leaf stage, for a total of 16.5 kBq per experimental unit. Glyphosate-tolerant canola plants, known to metabolize glyphosate into aminomethylphosphonate (AMPA) and glyoxylate,[22] were used as positive controls. Plants were harvested at 0, 24, 48 and 72 HAT with a total of three replications per time point per *E. colona* line. At each time point, treated leaves were rinsed to quantify [^{14}C]-glyphosate absorption, and whole plants were ground to a fine powder with liquid nitrogen, a pestle and a mortar. Plant material was transferred to 50-mL Falcon tubes, followed by the addition of ultrapure water (5 mL). Samples were homogenized with manual shaking for 30 s, and sonicated for 45 min at 65 °C. Following sonication, samples were held at room temperature for 30 min. Dichloromethane (10 mL) was added to

each tube and vigorously shaken for 1 min. A centrifugation step was carried out for 60 min at 3800*g* to obtain separate layers and facilitate the collection of an aliquot (4 mL) from the aqueous layer containing [^{14}C]-glyphosate. Collected aliquots were filtered with a 0.45 μm polyvinylidene fluoride (PVDF) syringe filter (Millex-HV, EMD Millipore, Tullagreen, Co, Cork, Ireland) into 20 mL scintillation vials, followed by a concentration step to dryness. An additional aliquot (0.5 mL) was also collected from Falcon tubes at this step for quantification of radiation mass balance with liquid scintillation spectrometry. Hence, [^{14}C]-glyphosate was reconstituted with 25 mM potassium dihydrogen phosphate (KH_2PO_4) (500 μL) and solutions were transferred to 2 mL injection vials before HPLC analysis. The HPLC system (1200 Infinity LC, Agilent, Santa Clara, CA, USA) was equipped with a Zorbax SAX column (4.6 × 250 mm, 5 μm), in line with a flow-through radioactivity detector (FlowStar LB 513, Berthold Technologies, Bad Wildbad, Germany). The mobile-phase (MP) A and B were composed of 25 and 75 mM KH_2PO_4, respectively. A gradient elution program was adopted as follows: 100% MP A for 7 min, a gradient increase to 100% MP B in 1 min, 100% MP B for 42 min, in a total run time of 60 min. Elution of [^{14}C]-AMPA occurred at 6 min, whereas elution of [^{14}C]-glyphosate occurred at 12.5 min.

2.3 Target site resistance

2.3.1 Shikimic acid accumulation assay

Shikimic acid accumulation was monitored over time as a proxy for EPSPS glyphosate sensitivity. The methods adopted were described by Alarcón-Reverte *et al.*[6] following methods by Cromartie and Polge,[23] with modifications. In brief, plants at the 3–4 leaf stage were treated with 0, 435 and 870 g a.e. ha^{-1} glyphosate (Roundup Powermax) using the previously described spray chamber. Youngest fully expanded leaves were harvested at 1, 24, 48 and 72 HAT. Fresh weight was recorded and tissue was homogenized in liquid nitrogen with two 3 mm grinding balls in a bead mill (Retsch MM300, Hann, Germany). Hydrochloric acid (0.25 M, 1 mL) was added to each sample and centrifuged at 3800*g* in a fixed arm rotor for 15 min. An aliquot of the supernatant (25 μL) was transferred to a 96 well flat-bottomed microtiter plate containing periodic acid solution (25% *w/v* periodic acid, 25% *w/v* sodium *m*-periodate) and incubated at room temperature for 1 h. The reaction was stopped with the addition of quench solution (0.6 M sodium hydroxide, 0.22 M sodium sulfite) and absorbance recorded at 380 nm using a MRXII microplate reader with Endpoint software (Dynex Technologies Inc., Chantilly, VA, USA). A standard curve of known shikimic acid concentrations across the experimental range was used to calculate the amount of shikimic acid in experimental samples. Five biological replicates and three technical replicates were used for each line at each time point and rate. The experiment was conducted twice.

2.3.2 Sequencing of EPSPS genomic DNA

Leaf tissue was harvested from young glasshouse grown plants of each *E. colona* line. DNA was extracted using DNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out using Platinum™ *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) in a total reaction volume (25 μL) containing 1X PCR buffer, 1.5 mM magnesium chloride ($MgCl_2$), 0.2 mM dNTP mix (Invitrogen), 0.2 μM of each primer and 400–500 ng template DNA. The primers EcLF 5′-AAGGACGCCAAAGAGGAAGT -3′ and EcLR 5′-ATCCCCTTGACACGAACAGG -3′ were used to produce a 475 bp amplicon covering Pro102 and Pro106 amino acid sites. PCR was performed in a thermal cycler with the following program: 5 min at 94 °C, 36 cycles of 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C, and a final extension for 7 min at 72 °C. The resulting fragments were ExoSAP treated using the ExoSAP-IT PCR Product Cleanup kit (Affymetrix, Santa Clara, CA, USA) as per manufacturer's instructions. Samples were then directly sequenced using the EcLF primer and BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

2.4 Data analysis

All data analysis was carried out in R version 3.3.3.[24] The dose–response mortality data were analyzed using a binomial two-parameter log-logistic model (Eqn 1) in the package 'drc'[25] where parameter *e* represents the LD_{50}, the rate that yields 50% mortality.

$$f(x) = \frac{1}{1 + \exp((\log(x) - \log(e)))} \quad (1)$$

Shikimic acid accumulation was fitted to a three-parameter log-logistic model (Eqn 2) using the package 'drc'.[25]

$$f(x) = \frac{d}{1 + \exp(b(\log(x) - \log(e)))} \quad (2)$$

Where the lower limit is set at 0 for the three-parameter model, *d* the upper limit, *e* represents the LD_{50} and *b* gives the relative slope. Absorption of [^{14}C]-glyphosate was analyzed using a rectangular hyperbolic model (Eqn 3) as described in Kniss *et al.*[26] using the package 'drc'.[25]

$$Y = (A_{max} \times T) / [(10/\theta) \times T_\theta + T] \quad (3)$$

Where *Y* is absorption expressed as percentage of total applied [^{14}C]-glyphosate, A_{max} is the maximum absorption, *t* is time and θ represents a percentage of *t* which here was set to $\theta = 90\%$. Translocation of [^{14}C]-glyphosate was analyzed using Eqn 3 where *Y* is the percent found in roots and non-treated leaves of total applied. The value A_{max} is substituted with T_{max}, representing the maximum translocation into the root and non-treated leaf samples.

3 RESULTS

3.1 Plant material and phenotype characterization

To minimize genetic variability, five single-seed-descendant lines were inbred. The lines were screened at the F_4 generation with a range of glyphosate concentrations and showed varying levels of resistance (Table 1). The LD_{50} values for susceptible populations A3 and C6 were 244.1 and 202.2 g a.e. ha^{-1} respectively, approximately 25% of the recommended field rate of 870 g a.e. ha^{-1}. Glyphosate rates lower than 435 g a.e. ha^{-1} provided 100% mortality of susceptible lines. The mortality-based resistance indices (RIs) for the resistant populations ranged from 2.4-fold (line H1) to 7.9-fold (line SV2). The GR_{50} RI based on aboveground biomass ranged from 1-fold (line A8) to 6.3-fold (line N3). The growth of *E. colona* line A8 at 21 days after treatment (DAT) was similar to that of susceptible lines by glyphosate when treated with a rate of 121.4 g a.e. ha^{-1}, however, this rate did not result in mortality of line A8. These results support previous dose response work on several of the field-collected parental populations.[19]

Table 1. Parameter estimates and resistance indices for the log-logistic equations used to calculate LD_{50} and GR_{50} of F_4 generation *Echinochloa colona* lines originating from orchard cropping systems of California in response to a range of foliar glyphosate treatments. Lines A3 and C6 are designated as susceptible to glyphosate and lines A8, H1, H5, N3 and SV2 resistant

Line	Mortality			Biomass		
	b	LD_{50}†(g a.e. ha^{-1})	RI	*b*	GR_{50}† (g a.e. ha^{-1})	RI
A3	3.83	244.1 ± 18.7	1.0	2.1	115.4 ± 3.3	1
A8	3.85	960.5 ± 79	3.9	0.4	121.4 ± 31	1
C6	3.28	202.2 ± 20.2	0.8	1.7	62.3 ± 2	0.5
H1	1.94	586.9 ± 89.4	2.4	1	218 ± 29	1.8
H5	2.73	852.5 ± 79.5	3.4	1.4	248 ± 17	2.1
N3	1.4	1584.2 ± 205	6.4	0.9	736 ± 121	6.3
SV2	1.26	1940.2 ± 257	7.9	1.6	621 ± 47	5.3

†Value (mean ± standard error).
Note: *b*, slope estimate; RI, resistance index relative to susceptible line A3. Data pooled from two experimental runs, $n = 33$.

Table 2. Parameter estimates and standard errors (SEs) for the rectangular hyperbola equations used to calculate total absorption (A_{max}) and rate of absorption (T90) of [^{14}C]-glyphosate as well as translocation (T_{max}) and rate of translocation (T90) for radiolabel applied to F_4 generation *Echinochloa colona* lines originating from orchard cropping systems of California. Lines A3 and C6 are designated as susceptible to glyphosate and lines A8, H1, H5, N3 and SV2 resistant

Absorption

Line	Phenotype	Parameter estimates			
		A_{max}†(%)	SE	T90‡(HAT)	SE
A3	S	31.5	2.3	39.4	16.4
A8	R	32.1	2.9	69.5	27.2
C6	S	33.9	4.3	103.8	51.8
H1	R	30.0	2.9	70.8	29.4
H5	R	25.9	2.6	48.5	26.1
N3	R	35.0	2.3	33.2	13.7
SV2	R	31.7	2.1	24.1	12.2

Translocation

		Parameter estimates			
		T_{max}†(%)	SE	T90‡ (HAT)	SE
A3	S	20.8	2.9	210.6	80.5
A8	R	20.5	6.8	396.9	278.1
C6	S	24.9	8.4	522.8	329.1
H1	R	17.1	3.6	249.1	133.5
H5	R	27.5	8.8	752.4	379.5
N3	R	25.3	6.8	407.3	226.2
SV2	R	18.8	2.9	198.8	85.7

†A_{max} denotes maximum [^{14}C]-glyphosate absorption expressed as a percentage of total applied.
‡T90 denotes time in hours to reach 90% of total [^{14}C]-glyphosate absorption.
§T_{max} denotes maximum radiolabel detected in roots and non-treated leaves as expressed as a percentage of total applied, SE denotes the standard error of the mean, HAT denotes hours after treatment with [^{14}C]-glyphosate.

3.2 Non-target site resistance

3.2.1 *Absorption and translocation of [^{14}C] glyphosate*

Absorption of [^{14}C]-glyphosate was similar among all tested *E. colona* lines. Maximum [^{14}C] absorption (A_{max}) ranged from 25.9 to 35%, but these values were not significantly different from the susceptible lines (31.5–33.9%) (Table 2). The T90 (time to absorption of 90% of total applied radiation) ranged from 24 to 103 HAT among the seven *E. colona* lines with no significant differences among resistant and susceptible lines.

Movement of [^{14}C] out of the treated leaf was observed over the 72 h time period in all lines tested, with no significant differences between resistant and susceptible *E. colona* lines (Table 2).

3.2.2 *Glyphosate metabolism*

The elution of [^{14}C]-glyphosate was detected at 4.5 min after sample injection in standards and positive controls. All *E. colona* lines showed the same elution time indicating no differences between resistant and susceptible lines (data not shown). The single [^{14}C] peak indicates no glyphosate metabolism in these plants. Recently, a new methodology has been used in two separate studies to detect glyphosate conversion to AMPA in resistant biotypes of *Digitaria insularis* (L.) Fedde and *Conyza canadensis* L. Cronq., other methods have not been reported to confirm metabolites.[27,28]

3.3 Target site resistance

3.3.1 *Shikimic acid accumulation*

Shikimic acid accumulated after glyphosate exposure in all tested lines, although accumulation was higher in susceptible lines (Fig. 1). Greater levels of shikimic acid accumulation were observed for lines A3 and C6 with 0.018 μg mg^{-1} fresh weight at 72 HAT (Fig. 1). The resistant line SV2 had the lowest level of shikimic acid accumulation in both glyphosate treatments with a maximum accumulation of 0.0039 μg mg^{-1} fresh weight, five-fold less than the susceptible line. When treated with 435 g a.e. ha^{-1}, all of the resistant lines accumulated significantly less shikimic acid compared to the susceptible lines. Similarly, at 870 g a.e. ha^{-1}, all but one resistant line accumulated less shikimic acid than the susceptible lines. The exception being line A8 which accumulated shikimic acid similar to the susceptible populations at that rate. This anomaly supports our previous observation of reduced growth and low GR_{50} (Table 1) of line A8 where growth was stunted compared to other resistant lines but survived better than the susceptible lines.

3.3.2 *EPSPS gene sequencing*

A 475 bp region of the *EPSPS* gene containing Pro102 and Pro106 sites was successfully amplified and sequenced from all *E. colona* lines. The resultant amplicons showed high sequence similarity to previously published *EPSPS* accessions with 96% similarity to *Sorghum halepense* (GenBank accession HQ436353)[29] and 95% similarity to *Zea mays* (GenBank accession X63374). Alignment of the *EPSPS* sequences show all resistant lines contained an amino acid change at Pro106 and susceptible lines contained the wild type codon CCA at position 106, encoding a proline (Table 3). No differences in amino acid at position 102 were observed among the lines tested, regardless of glyphosate sensitivity. Lines H1 and H5, derived from populations collected in Madera County, contained single nucleotide polymorphisms (SNPs) resulting in TCA (H1) and CTA (H5) producing amino acid changes from proline to serine and proline to leucine, respectively. Line A8, originating

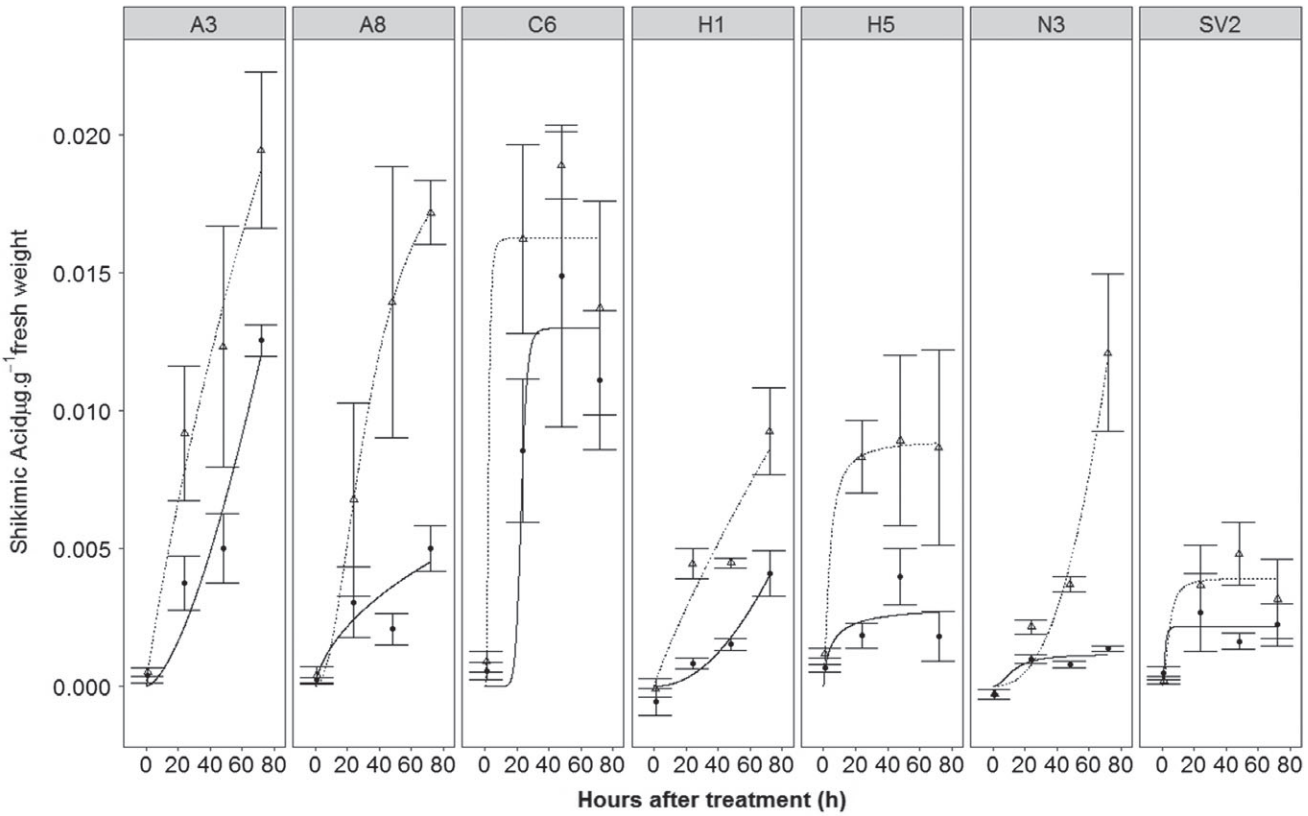

Figure 1. Shikimic acid accumulation in *Echinochloa colona* plants following the application of 435 (—●—) or 870 (—▲—) g a.e. ha^{-1} glyphosate. Data points are means ± standard error (SE) for $n = 10$. Lines A3 and C6 are designated as susceptible to glyphosate and lines A8, H1, H5, N3 and SV2 resistant.

from Butte County, contains two different SNPs at 106, ACA encoding a proline to threonine as well as CTA proline to leucine substitution. Lines N3 and SV2 originating from Kern County, contained SNPs (CTC) encoding a proline to leucine substitution. These collection locations span more than 500 km north to south in the Central Valley of California an area of intense agricultural production, particularly of orchards and annual crops.

4 DISCUSSION

The initial screening of field-collected *E. colona* populations from California orchards confirmed multiple populations that were able to survive treatment with a range of glyphosate rates, where LD_{50} values in these orchard collections ranged from 1520 to 2210 g a.e. ha^{-1}.[19] In this study on the F_4 generation lines, LD_{50} values ranged from 586 to 1940 g a.e. ha^{-1}. Previous reports of glyphosate resistance in *E. colona* have shown LD_{50} values that range between 229 and 1440 g a.e. ha^{-1} in populations from north-eastern Australia[30,31] and 80% survival at 840 g a.e. ha^{-1} in a population from California.[6] The range of resistance levels reported in *E. colona* worldwide and among the populations screened in this study may reflect the role of different mechanisms contributing to resistance. For example, reduced translocation of glyphosate in the plant generally confers a higher level of resistance than is observed with target site mutations[32] and more than one mechanism of resistance may be present within a plant.[30]

Absorption and movement of glyphosate within the plant was similar among resistant and susceptible plants in this study and, thus, was not considered a primary mechanism of resistance in these *E. colona* lines. Altered translocation of glyphosate has not been observed as a mechanism of resistance in *Echinochloa* species to date, despite being identified in other weed genera including *Conyza*,[21] *Lolium*[33] and *Chloris*.[15]

The accumulation of shikimic acid in all *E. colona* lines following treatment with glyphosate suggests that glyphosate is successfully reaching the chloroplast and that at least some portion of the EPSPS enzyme present is sensitive to the herbicide.[34,35] The varying

Table 3. Nucleotide and amino acid sequences of *EPSPS* cDNA sequenced from glyphosate-susceptible and -resistant F_4 *Echinochloa colona* lines derived from California orchard populations. Lines A3 and C6 are designated as susceptible to glyphosate and lines A8, H1, H5, N3 and SV2 resistant

Line	*EPSPS* codons†			
	102		106	
	Codon	Amino Acid	Codon	Amino Acid
A3	ACA‡	Threonine	CCA§	Proline
A8	ACA	Threonine	ACA CTA	Threonine Leucine
C6	ACA	Threonine	CCA	Proline
H1	ACA	Threonine	TCA	Serine
H5	ACA	Threonine	CTA	Leucine
N3	ACA	Threonine	CTC	Leucine
SV2	ACA	Threonine	CTC	Leucine

†Codon numbering from *Arabidopsis thaliana EPSPS* sequence (GenBank accession AT2G45300).
‡Wild-type genotype for codon 102.
§Wild-type genotype for codon 106.

levels of shikimic acid accumulation in these lines is consistent with an altered sensitivity of the EPSPS target site to glyphosate and/or differential exposure to glyphosate due to the amount reaching the target site.[6,36] Here we found five *E. colona* lines that accumulated significantly more shikimic acid when treated with the field rate of glyphosate compared to one-half that rate. Changes to the target site affects the sensitivity of EPSPS to glyphosate by causing a structural change at the binding site, and has been documented in many plant species as a mechanism of glyphosate resistance.[13]

Previous studies on glyphosate resistant populations of *E. colona* have detected target site mutations (TSMs) at Pro106 of the *EPSPS* gene. A Pro106Ser substitution was present in resistant *E. colona* populations from a maize field in California[6,37] and from Australia[38] which showed RIs of 6.6, 2.1 and 4.7 respectively. A Pro106Thr identified in a population from California[37] and Australia[30] resulted in RIs of 3.9 and 2.2 respectively and a Pro106Leu/Thr in a population from Australia had an RI of 1.28.[30]

A Pro106 mutation usually provides low to moderate levels of resistance compared to other mechanisms[30,39] which is consistent with the RI observed for the *E. colona* lines in this study.

This moderate level of resistance due, at least in part to TSM, is sufficient for plant survival at the recommended field rates of glyphosate in these populations. A Pro106Leu/Thr mutation in *E. colona* from Australia did not endow resistance to glyphosate at the field rate[30] whereas line A8 in the present study with a similar dual Pro106 mutation was stunted but had relatively high survival. One explanation of these differences may be the lines here have other mechanisms providing resistance. *Echinochloa colona* is an allopolyploid and the lines studied here were confirmed as hexaploid. The expression and overall allelic contribution of resistance: susceptible alleles in hexaploid *E. colona* is also not well understood and may have an important contribution to resistance levels observed in these lines. These plants contain at least six copies of EPSPS which may be homozygous or heterozygous between homologous or homeologous alleles. The overall allelic contribution of each gene copy, containing a TSM or not, is an important factor when considering resistance due to TSMs in polyploid species. This can be further complicated by differing transcriptional control between genomes which is well documented in polyploids (see Birchler *et al.*[40] for a general review of this work). Here we observed that, although line A8 contains two TSMs (Pro106Thr and Pro106Leu); these plants accumulated shikimic acid and had GR_{50} values similar to susceptible plants when treated with 870 g a.e. ha^{-1}. The observed differences between the three *E. colona* lines with only the Pro106Leu mutation and A8 (two TSMs, including Pro106Leu) could also be related to which genome that substitution occurs. Line SV2, which contains only the Pro106Leu mutation but was markedly different than H5 with the same mutation, accumulated the least shikimic acid at the field rate suggesting additional contributing mechanisms are likely present in some of these lines.

This work shows that glyphosate resistance in *E. colona* is present in multiple populations across the Central Valley of California. The presence of several different TSMs suggests these populations have evolved resistance independently of each other because of high selection pressure from the repeated use of glyphosate. Multiple mechanisms, including non-target site resistance (NTSR) may also be contributing to the variation in resistance between these *E. colona* lines that all contain a TSM, some with the same TSM. Temperature has been shown to affect resistance to glyphosate in *E. colona*[38] although the mechanisms are not well understood, as well as irradiance, humidity and water availability.[41] Further studies toward understanding the variation in resistance between these populations and the mechanisms underlying this will provide valuable information toward mitigating the further spread of resistance in this species. Little is known about the relative genomic contributions of resistance loci in weedy polyploid plants and how this may relate to fitness cost and/or adaptation to changing environments beyond resistance to this particular herbicide. Further characterization of *Echinochloa* species with different resistance mechanisms presents a unique opportunity to investigate additional weediness traits in polyploids that may contribute to their ability to survive across a wide range of environmental conditions with a great range of genetic plasticity. This will be of particular interest in forwarding our knowledge of the complexities of resistance in polyploid weed species and our ability to manage them in different farming systems.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Douglas Cook, Department of Plant Pathology, and Drs Jorge Dubcovsky and Marie Jasieniuk, Department of Plant Sciences at the University of California Davis, who facilitated equipment for molecular work. Funding for this research was provided by the California Department of Food and Agriculture Specialty Crop Block Grant Program, SCB13057.

REFERENCES

1 Holm L, Pucknett D, Pancho J and Herberger J, *The world's Worst Weeds: Distribution and Biology*. University Press of Hawaii Honolulu; 621 p, Honolulu, HI (1977).
2 Moretti M, Shrestha A, Hembree K and Hanson B, Postemergence control of glyphosate/paraquat-resistant hairy fleabane (*Conyza bonariensis*) in tree nut orchards in the Central Valley of California. *Weed Technol* **29**:501–508 (2016).
3 CDFA. *California Agricultural Production Statisitcs: California Department of Food and Agriculture* (2017). www.cdfa.ca.gov/statistics/ [accessed 20 December 2017].
4 Gaines TA, Cripps A and Powles SB, Evolved resistance to glyphosate in junglerice (*Echinochloa colona*) from the tropical Ord River region in Australia. *Weed Technol* **26**:480–484 (2012).
5 Heap I. *The International Survey Of Herbicide Resistant Weeds*. (2017). www.weedscience.org [accessed 12 December 2017].
6 Alarcón-Reverte R, García A, Urzúa J and Fischer AJ, Resistance to glyphosate in junglerice (*Echinochloa colona*) from California. *Weed Sci* **61**:48–54 (2013).
7 Funke T, Yang Y, Han H, Healy-Fried M, Olesen S, Becker A *et al.*, Structural basis of glyphosate resistance resulting from the double mutation Thr97 -> Ile and Pro101 -> Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. *J Biol Chem* **284**:9854–9860 (2009).
8 Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA and Dill GM, Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-Enolpyruvylshikimate-3-Phosphate Synthase. *Plant Physiol* **129**:1265–1275 (2002).
9 Howe AR, Gasser CS, Brown SM, Padgette SR, Hart J, Parker GB *et al.*, Glyphosate as a selective agent for the production of fertile transgenic maize *(Zea mays* L.) plants. *Mol Breed* **10**:153–164 (2002).
10 Kahrizi D, Salmanian AH, Afshari A, Moieni A and Mousavi A, Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5-enolpyruvylshikimate-3-phosphate synthase gene of *E. coli* (k12) and transformation of rapeseed (*Brassica napus* L.) in order to make tolerance to glyphosate. *Plant Cell Rep* **26**:95–104 (2007).
11 Han H, Vila-Aiub MM, Jalaludin A, Yu Q and Powles SB, A double EPSPS gene mutation endowing glyphosate resistance shows a remarkably high resistance cost. *Plant Cell Environ* **40**:3031–3042 (2017).
12 Gaines TA, Zhang W, Wang D, Bukun B, Chisholm ST, Shaner DL *et al.*, Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. *Proc Natl Acad Sci* **107**:1029–1034 (2010).

13 Sammons RD and Gaines TA, Glyphosate resistance: state of knowledge. *Pest Manag Sci* **70**:1367–1377 (2014).
14 Jasieniuk M, Ahmad R, Sherwood AM, Firestone JL, Perez-Jones A, Lanini WT *et al.*, Glyphosate-resistant italian ryegrass (*Lolium multiflorum*) in California: distribution, response to glyphosate, and molecular evidence for an altered target enzyme. *Weed Sci* **56**:496–502 (2008).
15 Brunharo CACG, Patterson EL, Carrijo DR, de Melo MSC, Nicolai M, Gaines TA *et al.*, Confirmation and mechanism of glyphosate resistance in tall windmill grass (*Chloris elata*) from Brazil. *Pest Manag Sci* **72**:1758–1764 (2016).
16 Ge X, d'Avignon DA, Ackerman JJH, Collavo A, Sattin M, Ostrander EL *et al.*, Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (*Lolium spp.*) from Australia, South America, and Europe: a 31P NMR Investigation. *J Agric Food Chem* **60**:1243–1250 (2012).
17 Yuan JS, Abercrombie LLG, Cao Y, Halfhill MD, Zhou X, Peng Y *et al.*, Functional genomics analysis of horseweed (*Conyza canadensis*) with special reference to the evolution of non-target-site glyphosate resistance. *Weed Sci* **58**:109–117 (2010).
18 Ge X, d'Avignon D, Ackerman J, Ostrander E and Sammons D, Application of 31P NMR spectroscopy to glyphosate studies in plants: insights into cellular uptake and vacuolar sequestration correlated to herbicide resistance, in *Handbook on Herbicides: Biological Activity, Classification and Health and Environmental Implications*, ed. by Kobayashi D and Watanabe E. Nova Science Publishers, Inc., Hauppauge, NY, pp. 55–83 (2014).
19 Moretti M, Garcia A, Fischer A and Hanson B, Distribution of glyphosate-resistant junglerice (*Echinochloa colona*) in perennial crops of the Central Valley of California. *Proceedings Western Society of Weed Science* **66**:24–25 (2013).
20 Buhler DD, Hoffman ML. *Andersen's Guide to Practical Methods of Propagating Weeds & Other Plants: Weed Science Society of America*; 1999.
21 Moretti ML and Hanson BD, Reduced translocation is involved in resistance to glyphosate and paraquat in *Conyza bonariensis* and *Conyza canadensis* from California. *Weed Res* **57**:25–34 (2017).
22 Barry GF and Kishore GM, inventors, *Glyphosate tolerant plants*. Monsanto Company Assignee, United States (1995).
23 Cromartie T and Polge N, An improved assay for shikimic acid and its use as a monitor for the activity of sulfosate. *Proc Weed Sci Soc Am* **40**:291 (2000).
24 R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (2018).
25 Ritz C, Streibig J. *Package 'drc'. CRAN*. (2015). http://www.r-project.org, http://www.bioassay.dk [accessed 15 December 2017].
26 Kniss A, Vassios J, Nissen S and Ritz C, Nonlinear regression analysis of herbicide absorption studies. *Weed Sci* **59**:601–610 (2011).
27 de Carvalho LB, Alves PLCA, González-Torralva F, Cruz-Hipolito HE, Rojano-Delgado AM, De Prado R *et al.*, Pool of resistance mechanisms to glyphosate in Digitaria insularis. *J Agric Food Chem* **60**:615–622 (2012).
28 González-Torralva F, Rojano-Delgado AM, Luque de Castro MD, Mülleder N and De Prado R, Two non-target mechanisms are involved in glyphosate-resistant horseweed (Conyza canadensis L. Cronq.) biotypes. *J Plant Physiol* **169**:1673–1679 (2012).
29 Vila-Aiub MM, Balbi MC, Distéfano AJ, Fernández L, Hopp E, Yu Q *et al.*, Glyphosate resistance in perennial *Sorghum halepense* (johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. *Pest Manag Sci* **68**:430–436 (2012).
30 Han HP, Yu Q, Widderick MJ and Powles SB, Target-site EPSPS Pro-106 mutations: sufficient to endow glyphosate resistance in polyploid *Echinochloa colona*? *Pest Manag Sci* **72**:264–271 (2016).
31 Goh SS, Vila-Aiub MM, Busi R and Powles SB, Glyphosate resistance in *Echinochloa colona*: phenotypic characterisation and quantification of selection intensity. *Pest Manag Sci* **72**:67–73 (2016).
32 Cross RB, McCarty LB, Tharayil N, McElroy JS, Chen S, McCullough PE *et al.*, A Pro(106) to Ala substitution is associated with resistance to glyphosate in annual bluegrass (*Poa annua*). *Weed Sci* **63**:613–622 (2015).
33 Fernandez-Moreno PT, Bastida F and De Prado R, Evidence, mechanism and alternative chemical seedbank-level control of glyphosate resistance of a rigid ryegrass (*Lolium rigidum*) biotype from southern Spain. *Front Plant Sci* **8**:450 (2017).
34 Duke SO and Powles SB, Glyphosate: a once-in-a-century herbicide. *Pest Manag Sci* **64**:319–325 (2008).
35 Shaner D, Testing methods for glyphosate resistance, in *Glyphosate Resistance in Crops and Weeds: History, Development, and Management*, ed. by Nandula V. John Wiley & Sons, Hoboken, NJ, pp. 93–118 (2010).
36 Powles S and Preston C, Evolved glyphosate resistance in plants: biochemical and genetic basis of resistance. *Weed Technol* **20**:282–289 (2006).
37 Alarcon-Reverte R, Garcia A, Watson SB, Abdallah I, Sabate S, Hernandez MJ *et al.*, Concerted action of target-site mutations and high EPSPS activity in glyphosate-resistant junglerice (*Echinochloa colona*) from California. *Pest Manag Sci* **71**:996–1007 (2015).
38 Nguyen TH, Malone JM, Boutsalis P, Shirley N and Preston C, Temperature influences the level of glyphosate resistance in barnyardgrass (*Echinochloa colona*). *Pest Manag Sci* **72**:1031–1039 (2016).
39 Kaundun SS, Zelaya IA, Dale RP, Lycett AJ, Carter P, Sharples KR *et al.*, Importance of the P106S target-site mutation in conferring resistance to glyphosate in a goosegrass (*Eleusine indica*) population from the Philippines. *Weed Sci* **56**:637–646 (2008).
40 Birchler JA, Genetic consequences of polyploidy in plants, in *Polyploidy and Genome Evolution*, ed. by Soltis DE and Soltis PS. Springer, Berlin (2012).
41 Adkins SW, Tanpipat S, Swarbrick JT and Boersma M, Influence of environmental factors on glyphosate efficacy when applied to *Avena fatua* or *Urochloa panicoides*. *Weed Res* **38**:129–138 (1998).

