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# *EPSPS* gene amplification confers glyphosate resistance in Palmer amaranth in Connecticut

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## Research Article

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## Abstract

A Palmer amaranth biotype (CT-Res) with resistance to glyphosate was recently confirmed in a pumpkin field in Connecticut. However, the underlying mechanisms conferring glyphosate resistance in this biotype is not known. The main objectives of this research were 1) to determine the effect of plant height (10, 20, and 30 cm) on glyphosate resistance levels in CT-Res Palmer amaranth biotype, and 2) to investigate whether the target site–based mechanisms confer glyphosate resistance. To achieve these objectives, progeny seeds of the CT-Res biotype after two generations of recurrent selection with glyphosate (6,720 g ae $ha^{-1}$) were used. Similarly, known glyphosate-susceptible Palmer amaranth biotypes from Kansas (KS-Sus) and Alabama (AL-Sus) were included. Results from greenhouse dose-response studies revealed that CT-Res Palmer amaranth biotype had 69-, 64-, and 54-fold resistance to glyphosate as compared with the KS-Sus biotype when treated at heights of 10, 20, and 30 cm, respectively. Sequence analysis of the *EPSPS* gene revealed no point mutations at the $Pro_{106}$ and $Thr_{102}$ residues in the CT-Res Palmer amaranth biotype. Quantitative polymerase chain reaction analysis revealed that the CT-Res biotype had 33 to 111 relative copies of the *EPSPS* gene compared with the AL-Sus biotype. All these results suggest that the *EPSPS* gene amplification endows a high level of glyphosate resistance in the GR Palmer amaranth biotype from Connecticut. Because of the lack of control with glyphosate, growers should adopt the use of effective alternative preemergence and postemergence herbicides in conjunction with other cultural and mechanical tactics to mitigate the further spread of GR Palmer amaranth in Connecticut.

## Introduction

Palmer amaranth is one of the most troublesome summer annual weeds in most agronomic and non-crop production systems across southern, midwestern, and U.S. Great Plains regions (Aulakh et al. 2012, 2013, 2021; Bensch et al. 2003; Chahal et al. 2017; Crow et al. 2016; Grichar 1997: Meyers et al. 2010; Mohseni-Moghadam et al. 2013b; Norsworthy et al. 2008b; Price et al. 2006, 2011; Smith et al. 2000). An extended emergence period, $C_4$ photosynthetic pathway, high water-use efficiency, dioecious nature (separate male and female plants) of sexual reproduction, prolific seed production (100,000 to 1,000,000 seeds per plant), and a tendency to evolve herbicide resistance are the salient traits for rapid invasion and spread of Palmer amaranth into new regions (Burke et al. 2007; Ehleringer 1983; Horak and Loughin 2000; Keeley et al. 1987; Ward et al. 2013).

Glyphosate was commercialized in 1974 and was a highly efficacious postemergence (POST) herbicide for controlling Palmer amaranth (Corbett et al. 2004; Culpepper and York 1998; Parker et al. 2005). Glyphosate targets the 5-enolypyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme in the shikimic acid pathway of plants and microorganisms (della-Cioppa et al. 1986). The disruption of this pathway prevents the production of essential aromatic amino acids, including phenylalanine, tryptophan, tyrosine, and other important secondary metabolites that eventually lead to plant death (Duke and Powles 2008). Commercialization of glyphosate-resistant (GR) crops in the mid-1990s and its rapid adoption resulted in almost exclusive reliance on glyphosate for broad-spectrum weed control (Norsworthy et al. 2007). Due to the high effectiveness and relatively low cost of glyphosate-based weed control in GR crops, glyphosate eventually replaced the use of pre-plant incorporated (PPI), preemergence, selective POST, and post-directed (PD) herbicides and greatly increased the selection of GR weed biotypes (Young 2006). Within two decades of commercialization of GR crops, several weed species, including Palmer amaranth, were reported with resistance to glyphosate. First, a GR Palmer amaranth biotype was discovered in Macon County, GA, in 2004 (Culpepper et al. 2006).

Currently, GR Palmer amaranth biotypes have been confirmed in 30 U.S. states (Heap 2024). Some GR Palmer amaranth biotypes required 115 times higher glyphosate rate than susceptible biotypes to achieve 50% control (Norsworthy et al. 2008a; Steckel et al. 2008). Currently, resistance to 10 different herbicide sites of action (SOAs) has been identified in Palmer amaranth biotypes across the United States (Heap 2024), including inhibitors of acetolactate synthase (ALS; categorized as a Group 2 herbicide by the Weed Science Society of America [WSSA]), microtubule assembly (WSSA Group 3), photosystem II (PS II; WSSA Groups 5 and 6), EPSPS (WSSA Group 9), glutamine synthetase (WSSA Group 10), protoporphyrinogen oxidase (WSSA Group 14), very long-chain fatty acid elongase (WSSA Group 15), 4-hydroxyphenylpyruvate dioxygenase (WSSA Group 27), and synthetic auxins (WSSA Group 4) (Carvalho-Moore et al. 2022; Chahal et al. 2017; Culpepper et al. 2006; Foster and Steckel 2022; Gossett et al. 1992; Heap 2024; Jhala et al. 2014; Kouame et al. 2022; Kumar et al. 2019, 2020; Nakka et al. 2017; Priess et al. 2022; Salas et al. 2016; Sprague et al. 1997). Furthermore, Palmer amaranth biotypes resistant to multiple herbicide SOAs are present in several corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L. Merr.), and vegetable production systems in the United States (Aulakh et al. 2021; Heap 2024; Kouame et al. 2022; Kumar et al. 2019, 2020).

Weed species have evolved multiple mechanisms conferring glyphosate resistance (Chatham et al. 2015a; Dinelli et al. 2008; Perez-Jones et al. 2007; Shaner et al. 2011; Simarmata and Penner 2008; Wiersma et al. 2015). Most commonly reported glyphosate resistance mechanisms include target site mutation in the *EPSPS* gene (Baerson et al. 2002; Kaundun et al. 2011; Perez-Jones et al. 2007; Wakelin and Preston 2006; Yu et al. 2007), reduced absorption and translocation (Dinelli et al. 2008; Lorraine-Colwill et al. 2003; Wakelin et al. 2004; Yu et al. 2007), enhanced sequestration (Ge et al. 2010), and *EPSPS* gene amplification (Chahal et al. 2017; Chatham et al. 2015b; Gaines et al. 2010; Kumar et al. 2015). A GR Palmer amaranth biotype with >100 *EPSPS* gene copies has been reported from Georgia (Gaines et al. 2010). Furthermore, increased *EPSPS* gene copies have also been reported in GR Palmer amaranth biotypes from Mississippi (Ribeiro et al. 2014), Nebraska (Chahal et al. 2017), and New Mexico (Mohseni-Moghadam et al. 2013a).

GR Palmer amaranth has recently been reported in Connecticut (Aulakh et al. 2021). However, the mechanism of glyphosate resistance has not been characterized in that biotype. Thus, the main objectives of this research were 1) to determine the glyphosate resistance levels in GR Palmer amaranth biotype from Connecticut when treated at three different plant heights, and 2) to determine whether one or more target site–based mechanisms confers glyphosate resistance in the Connecticut biotype.

## Materials and Methods

### Plant Material

A confirmed GR Palmer amaranth biotype (CT-Res) from Hartford County, CT (41.93°N, 72.53°W), was investigated. In 2019, the GR plants that survived 6,720 g ae ha$^{-1}$ of glyphosate (MADDOG®; Loveland Products, Inc., Loveland, CO) in the previously reported whole-plant dose-response bioassay (Aulakh et al. 2021) were allowed to open-pollinate to develop an "$OP_1$" population. Seeds from female plants were harvested, cleaned thoroughly using a vertical air column blower, and stored in airtight polyethylene bags at 4 C until further testing. In 2022, seedlings from the "$OP_1$" population were treated again with glyphosate (6,720 g ae ha$^{-1}$), and the survivors were allowed to open pollinate to produce the "$OP_2$" seeds. Seeds from "$OP_2$" female plants were harvested, cleaned, and stored in airtight polyethylene bags at 4 C until further testing. A known glyphosate-susceptible biotype (KS-Sus) from the Kansas State University Agricultural Research Center near Hays, KS (38°50N, 99°18W), was used in the whole-plant dose response bioassays. Previous dose-response experiments confirmed that KS-Sus was highly susceptible to glyphosate with an $ED_{90}$ value of 424 g ae ha$^{-1}$ (Aulakh et al. 2021). Another known glyphosate susceptible biotype (AL-Sus) acquired from the E.V. Smith Research Center near Shorter, AL (32°26N, 85°56W), and the campus of Auburn University was used to determine the underlying target site–based mechanisms of glyphosate resistance.

### Effect of Plant Height on Glyphosate Resistance Levels

Whole-plant dose-response bioassays were conducted in the summer of 2023 in a greenhouse at the Connecticut Agricultural Experiment Station, Windsor, CT, to determine the response of CT-Res ("$OP_2$") Palmer amaranth biotype to glyphosate at three different plant heights (10, 20, and 30 cm). Seeds of both CT-Res ("$OP_2$") and KS-Sus biotypes were planted in square plastic pots (10 × 10 × 12 cm) containing planting media (Pro-Mix Premium All Purpose®; Quakertown, PA). Pro-Mix Premium All Purpose contains Canadian sphagnum peat moss (80% to 90%), peat humus, perlite, limestone, and mycorrhizae PTB297 technology. Palmer amaranth plants were thinned to one plant per pot at 7 d after emergence. The experiment was arranged in a randomized complete block (blocked by biotype) design with a 9 × 2 × 3 factorial arrangement of treatments. The three factors were 1) nine glyphosate rates: 0×, 0.125×, 0.25×, 0.5×, 1×, 2×, 4×, 8×, and 16×, where 1× is the field-use rate of glyphosate (840 g ae ha$^{-1}$); 2) two Palmer amaranth biotypes: CT-Res and KS-Sus; and 3) three plant heights: 10, 20, and 30 cm. Each factorial treatment combination was replicated six times (one plant per pot), and the experiment was repeated twice. The greenhouse was maintained at 30/26 C day/night temperatures with a 16-h photoperiod supplemented by overhead sodium halide lamps with light intensity of 450 µ mol s$^{-1}$. Plants were watered with an overhead sprinkler system as needed to avoid the moisture stress and maintain good growth. Palmer amaranth seedlings were treated with glyphosate (MADDOG®; Loveland Products, Inc., Loveland, CO), and each glyphosate treatment was prepared in distilled water mixed with a nonionic surfactant (Induce; Helena Chemical Co., Collierville, TN) at 0.25% vol/vol. Glyphosate treatments were applied with a compressed $CO_2$ backpack sprayer through a single TeeJet AI8002VS flat-fan spray nozzle (Spraying Systems Co., Wheaton, IL) calibrated to deliver 187 L ha$^{-1}$ spray volume at 207 kPa and 3.5 km h$^{-1}$. Plants were harvested at 21 d after treatment (DAT), and shoot fresh weight was determined. The fresh weights were then converted into percent biomass reduction compared with the nontreated control (Wortman 2014) as shown in Equation 1:

$$\text{Biomass reduction (\%)} = \frac{(\overline{C} - B)}{\overline{C}} \times 100 \qquad [1]$$

where $\overline{C}$ is the mean fresh weight biomass of the nontreated control and $B$ is the biomass of an individual treated plant.

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### Statistical Analysis

Due to nonsignificant interaction (P = 0.324) of treatment-by-run, data on fresh shoot biomass reduction (%) of both CT-Res and KS-Sus Palmer amaranth biotypes were averaged across two runs. A three-parameter log-logistic model (Eq. 2) was fitted on biomass reduction using the DRC package in R software (R Foundation for Statistical Computing, Vienna, Austria) (Knezevic et al. 2007):

$$Y = \frac{d}{1 + \exp\left[b(logx - loge)\right]} \quad [2]$$

where $Y$ is the percent fresh shoot biomass reduction, $x$ is the herbicide rate, $d$ is the upper limit, $e$ is the $GR_{50}$ value (amount of glyphosate needed for 50% reduction in fresh shoot biomass), and $b$ represents the relative slope around the parameter "$e$". The level of resistance was calculated by dividing the $GR_{90}$ value (amount of glyphosate needed for 90% reduction in fresh shoot biomass) of the resistant biotype (CT-Res) by that of the susceptible biotype (KS-Sus) for the corresponding plant height.

**Table 1.** Regression parameter estimates based on shoot fresh weight (% of nontreated) of a glyphosate-resistant Palmer amaranth population from Connecticut and a glyphosate-susceptible population from Kansas 21 d after treatment with various glyphosate doses[a,b]

| | | Parameter estimates (± SE) | | | | |
|---|---|---|---|---|---|---|
| Plant height | Biotype | *d* | *b* | $GR_{50}$ | $GR_{90}$ | R/S |
| cm | | | | | | |
| 10 | CT-Res | 100 (2.9) | 1.7 (0.3) | 5,138 | 18,05 | 69 |
| | KS-Sus | 99 (5.3) | 1.5 (0.2) | 74 | 326 | |
| 20 | CT-Res | 100 (1.8) | 1.5 (0.1) | 6,908 | 29,942 | 64 |
| | KS-Sus | 99 (3.2) | 1.1 (0.1) | 108 | 750 | |
| 30 | CT-Res | 102 (2.6) | 1.1 (0.2) | 13,221 | 100,716 | 54 |
| | KS-Sus | 99 (3.7) | 0.9 (0.2) | 247 | 2,251 | |

[a]Abbreviations: *b*, the relative slope around the $GR_{50}$ value; *d* the upper limit of biomass reduction; CT-Res, glyphosate-resistant Palmer amaranth biotype from Enfield, CT; KS-Sus, susceptible Palmer amaranth biotype from Hays, KS; $GR_{50}$, the effective dose (g ae $ha^{-1}$) of glyphosate needed for 50% fresh shoot weight reduction (% of nontreated); $GR_{90}$, the effective dose (g ae $ha^{-1}$) of glyphosate needed for 90% fresh shoot weight reduction (% of nontreated); R/S, resistance index (estimated as a ratio of $GR_{50}$ of a CT-Res to $GR_{50}$ of the KS-Sus Palmer amaranth biotype).
[b]Data were obtained from a greenhouse study conducted at the Connecticut Agricultural Experiment Station, Windsor, CT.

### Mechanism(s) of Glyphosate Resistance

#### Genomic DNA Isolation

The AL-Sus plants were grown using the same planting medium and greenhouse conditions previously mentioned in the whole-plant dose-response bioassays. Fresh leaf tissue was collected from the nontreated AL-Sus plants (two plants) and the CT-Res plants (six plants) that survived 6,720 g ae $ha^{-1}$ of glyphosate in the 2023 dose-response bioassay. The harvested leaf tissue (100 mg) was immediately flash-frozen in liquid nitrogen (−195.79 C) and stored at −80 C for genomic DNA (gDNA) isolation and extraction. The gDNA extraction was performed with the Wizard® Genomic DNA purification kit (Promega Corporation. Madison, WI) protocol for plant tissue. Quantification of extracted DNA was performed with a Nanodrop™ One C (Thermo Fisher Scientific, Waltham, MA).

#### Sequencing of EPSPS $Thr_{102}$ and $Pro_{106}$ Codons

The conserved region of the *EPSPS* gene encompassing $Pro_{106}$ and $Thr_{102}$ codons was amplified for the CT-Res and AL-Sus biotypes by polymerase chain reaction (PCR). The primers used in this experiment were obtained from *EPSPS* genomic sequences available on the National Center for Biotechnology Information database under accession MT025716.1. The primer set previously identified for Palmer amaranth *EPSPS* sequence (200 base pairs [bp]) was used (Gaines et al. 2010; Whaley et al. 2006): (forward) EPSF1, 5′-ATG TTG GAC GCT CTC AGA ACT CTT-3′ GGT; (reverse) EPSR8, 5′-TGA ATT TCC TCC AGC AAC GGC AA-3′. The PCR was performed with the DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific) using the following thermocycle conditions: an initial denaturation at 95 C for 1 min; 40 denaturation cycles at 95 C for 30 s, primer annealing at 52 C for 30 s, and extension at 72 C for 3 min. A final extension at 72 C for 10 min was included. Amplicons were visualized with electrophoresis (1% agarose). The amplicons were extracted from agarose gels with the Wizard® SV Gel and PCR Clean-Up System (Promega) and quantified spectrophotometrically as previously described. Samples were sent for Sanger sequencing at the Genomics Core Facility at the Pennsylvania State Huck Institute of Life Sciences. Sequencing primers were used to cover all single nucleotide polymorphisms (SNPs) known to confer glyphosate resistance (Heap 2024). Sequencing primers for *EPSPS* were EPSF1 and EPSPR8. Sequencing results were aligned and visually analyzed using Geneious Prime software (Biomatters Inc., Boston, MA). The *EPSPS* sequence of the CT-Res biotype was aligned to a reference AL-Sus biotype *EPSPS* sequence to determine substitutions at $Pro_{106}$ or $Thr_{102}$ codons.

#### EPSPS Genomic Copy Number

Genomic DNA was used to quantify the number of copies of the *EPSPS* gene in CT-Res plants relative to the *ALS* gene (housekeeping gene) with a real-time PCR (Quantum Studio 5; Thermo Fisher) and the Power Track™ SYBR™ Green Master Mix protocol (Thermo Fisher). Primers for the housekeeping gene were: (forward) ALSF2, 5′-GCT GCT GAA GGC TAC GCT-3′ and (reverse) ALSFR2, 5′-GCG GGA CTG AGT CAA GAA GTG-3′ for ALS amplification. *EPSPS* amplification primers were: (forward) ECC_EPSPS_F1, 5′-CCA GAC CAA ATA CTT TCG GA-3′ and (reverse) ECC_EPSPS_R2, 5′CGG TAT GCT TAG AGG TGA AA-3′ (Gaines et al. 2010). Three technical replicates and negative controls were also included. The real-time PCR conditions were as follows: enzyme activation at 95 C for 2 min, 40 cycles of denaturation at 95 C for 15 s, and 40 cycles of annealing and extension at 60 C for 1 min. A melt curve was produced to evaluate the specificity of the primers by setting the following conditions: First, a ramp rate of 1.6 C $s^{-1}$ increases the temperature gradually up to 95 C, holding it for 15 s. A second ramp rate 1.6 C $s^{-1}$ up to 60 C for 1 min was included, followed by a final dissociation step with a ramp rate of 0.075 C $s^{-1}$ up to 95 C for 15 s. The $2^{-\Delta\Delta Ct}$ method was used to quantify copy number variation of the *EPSPS* gene relative to the *ALS* gene. The *EPSPS* gene copies in CT-Res plants were assessed relative to a known glyphosate-susceptible biotype (AL-Sus, a calibrator sample). Data analysis was performed using R studio software by calculating the mean fold change per sample and further applying the least squares means comparison using the EMMEANS (Lenth 2022) package. Means comparison were performed using the MULTCOMP (Hothorn et al. 2008) package ($\alpha = 0.05$), and data were plotted using the GGPLOT2 (Wickham 2016) package.

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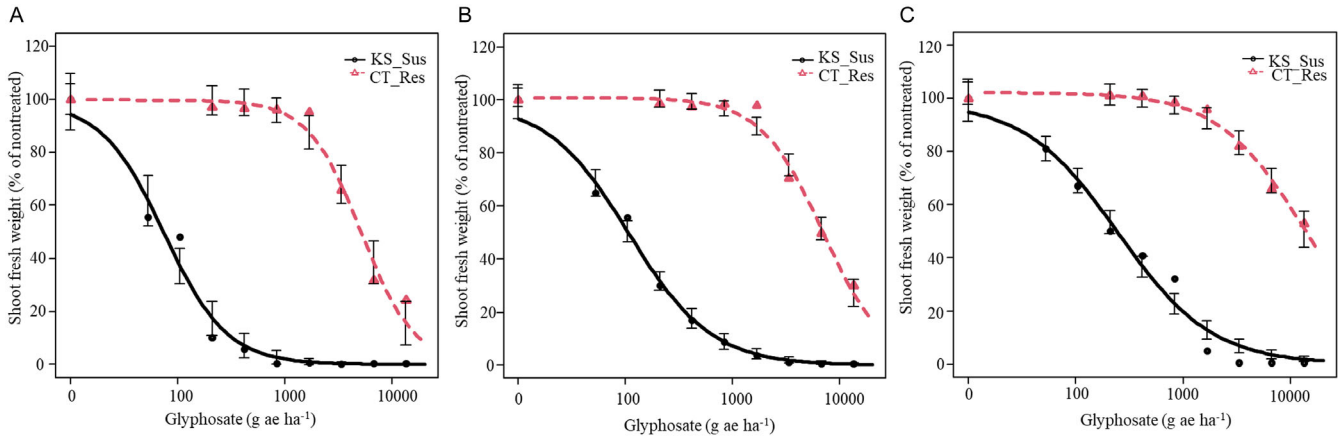


**Figure 1.** Glyphosate dose-response curves for 10-cm (A), 20-cm (B), and 30-cm (C) tall CT-Res and KS-Sus biotypes. CT-Res, resistant Palmer amaranth biotype found in Hartford County, Connecticut; KS-Sus, Palmer amaranth biotype collected from Kansas State University Agricultural Research Center near Hays, KS. Percent reduction in the shoot fresh biomass was calculated using Equation 1 in the text (Wortman 2014). A three-parameter log-logistic model was fitted on biomass reduction using Equation 2 in the text (Knezevic et al. 2007) using the DRC package (R statistical software; R Foundation for Statistical Computing, Vienna, Austria); AL-Sus1AL-Sus2CT-Res1CT-Res2CT-Res3CT-Res4CT-Res5CT-Res6AL-Sus1AL-Sus2CT-Res1CT-Res2CT-Res3CT-Res4CT-Res5CT-Res6.

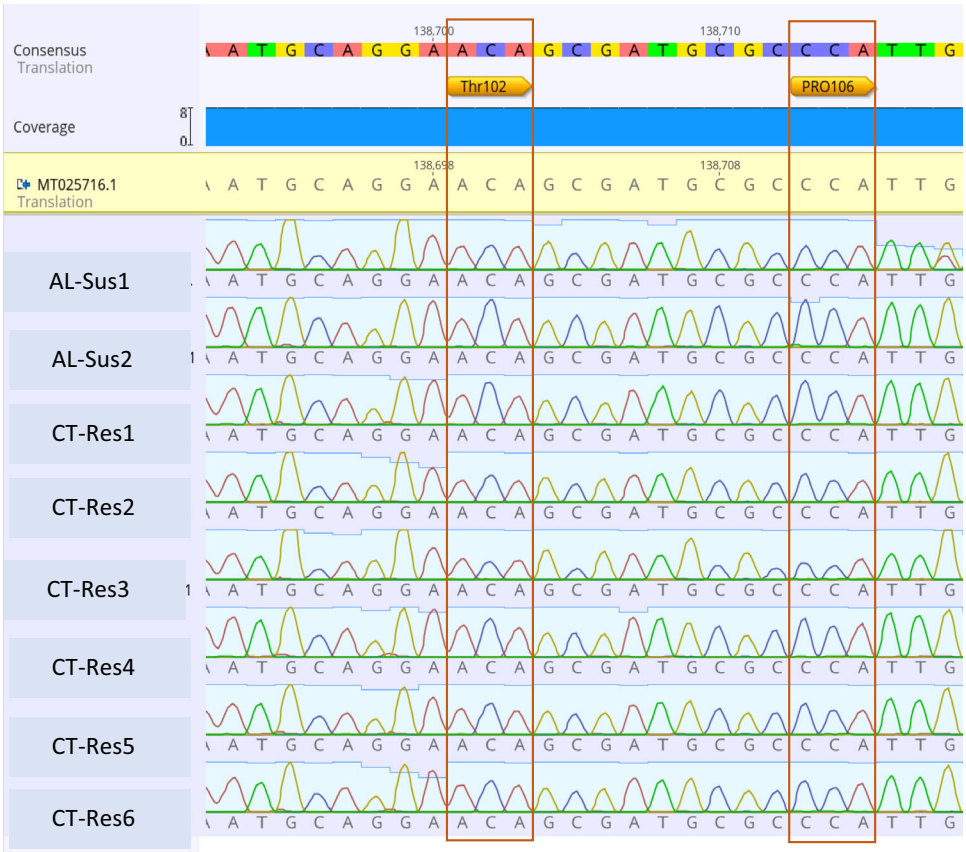


**Figure 2.** *EPSPS* gene sequence demonstrating no point mutations at the $Pro_{106}$ (amino acid substitution from proline to serine, threonine, alanine, or leucine) and $Thr_{102}$ (amino acid substitution from threonine to isoleucine) codons. AL-Sus1 and AL-Sus2 indicate glyphosate susceptible plants from Alabama; CT-Res1, CT-Res2, CT-Res3, CT-Res4, CT-Res5, and CT-Res6 indicate glyphosate-resistant plants from Connecticut.

## Results and Discussion

### *Effect of Plant Height on Glyphosate Resistance Levels*

The estimated rates of glyphosate required for a 50% reduction in shoot fresh weight ($GR_{50}$) of 10-, 20-, and 30-cm-tall CT-Res biotype were 5,138, 6,908, and 13,221 g ae $ha^{-1}$, respectively (Table 1, Figure 1, A, B, and C). In contrast, the corresponding $GR_{50}$ values for 10-, 20-, and 30-cm-tall KS-Sus biotype were 74, 108, and 247 g ae $ha^{-1}$, respectively. The reduction in shoot fresh weight of the CT-Res biotype with 840 g ae $ha^{-1}$ of glyphosate was below 10%, regardless of the plant height at the time of treatment. Glyphosate rates estimated for a 90% reduction in shoot fresh

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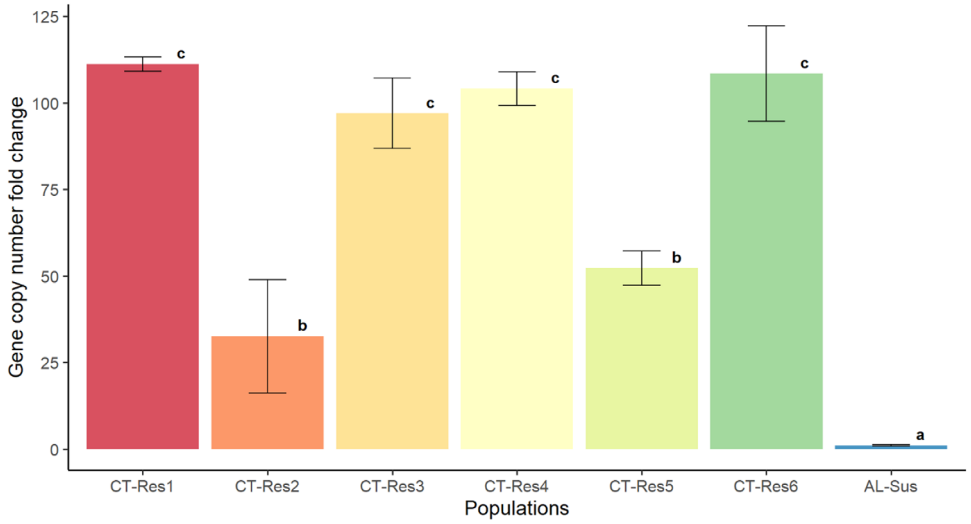


**Figure 3.** Bar plot of *EPSPS* gene copy number fold change relative to the *ALS* gene, obtained with the $2^{-\Delta\Delta Ct}$ method. The same letters indicate no significant difference among biotypes (P = 0.05). Error bars indicate standard deviation. AL-Sus indicates glyphosate-susceptible plants from Alabama; CT-Res1, CT-Res2, CT-Res3, CT-Res4, CT-Res5, and CT-Res6 indicate glyphosate-resistant plants from Connecticut.

weight ($GR_{90}$) were 18,056, 29,942, and 100,716 g ae $ha^{-1}$ for the CT-Res biotype plants treated at heights of 10, 20, and 30 cm, respectively (Table 1). Complete control of the CT-Res biotype was not achieved even at the highest rate of glyphosate (13,340 g ae $ha^{-1}$) tested in the dose-response bioassay. Similar $GR_{90}$ values have previously been reported for 10-cm-tall GR Palmer amaranth biotypes in Nebraska and Arkansas (Chahal et al. 2017; Norsworthy et al. 2008b). On the contrary, the KS-Sus plants up to 20 cm tall were at least 90% controlled with 840 g ae $ha^{-1}$ of glyphosate. However, the $GR_{90}$ value was much higher (2,251 g ae $ha^{-1}$) for 30-cm-tall KS-Sus plants. Several researchers found large differences in $GR_{50}$ and $GR_{90}$ values of susceptible and GR Palmer amaranth biotypes (Norsworthy et al. 2008a; Sosnoskie et al. 2011; York 2007). A GR Palmer amaranth biotype from Arkansas had an $I_{50}$ value of 2,800 g ae $ha^{-1}$ compared with 35 ae $ha^{-1}$ for the susceptible biotype (Norsworthy et al. 2008a). Sosnoskie et al. (2011) reported 50% control of the glyphosate-susceptible and GR biotypes with glyphosate rates of 91 and 103 g ae $ha^{-1}$, respectively. In the same study, ≥90% reduction in fresh weight was observed with glyphosate at 197 g ae $ha^{-1}$ and 2,363 g ae $ha^{-1}$ for the susceptible and GR Palmer amaranth biotypes, respectively. Several GR Palmer amaranth biotypes from North Carolina had $I_{50}$ values between 180 g ae $ha^{-1}$ and 360 g ae $ha^{-1}$, compared with 89 g ae $ha^{-1}$ for the local glyphosate-susceptible biotype (York 2007).

In this study, the CT-Res Palmer amaranth biotype exhibited 69-fold, 64-fold, and 54-fold resistance to glyphosate when plants were treated at heights of 10, 20, and 30 cm, respectively (Table 1). Aulakh et al. (2021) reported 10-fold resistance to glyphosate in the same CT-Res Palmer amaranth biotype compared with the KS-Sus biotype. However, it is important to note that whole-plant dose-response bioassay in an earlier study was conducted on GR Palmer amaranth plants propagated from a field-collected segregating biotype. In the current dose-response study, test plants were grown from $OP_2$ seeds of plants that survived 6,720 g ae $ha^{-1}$ of glyphosate herbicide. Similar levels of glyphosate resistance have also been reported for GR Palmer amaranth from Kansas, Mississippi, and Nebraska (Chahal et al. 2017; Kumar et al. 2019; Kumar et al. 2020; Nandula et al. 2012).

### *EPSPS* Gene Sequencing

The point mutations at the $Pro_{106}$ (amino acid substitution from proline to serine, threonine, alanine, or leucine) and $Thr_{102}$ (amino acid substitution from threonine to isoleucine) codons in the *EPSPS* gene have previously been reported to confer glyphosate resistance in some GR weed species (Sammons and Gaines 2014; Yu et al. 2015). However, the sequence analysis of the *EPSPS* gene revealed no point mutations at the $Pro_{106}$ and $Thr_{102}$ residues in the CT-Res Palmer amaranth plants (Figure 2). These results rule out the possibility of a point mutation at the $Pro_{106}$ or $Thr_{102}$ codons in the *EPSPS* gene for a possible mechanism of glyphosate resistance in the CT-Res biotype. Lack of target-site mutations conferring glyphosate resistance has also previously been reported in GR kochia [*Kochia scoparia* (L.) Schrad], Palmer amaranth, and spiny amaranth biotypes (Gaines et al. 2010; Kumar et al. 2015; Nandula et al. 2014).

### *EPSPS* Gene Amplification

The *EPSPS* gene amplification (increased copy number) has previously been reported in various GR weed biotypes (Chatham et al. 2015b). The qPCR analysis indicated that plants of CT-Res Palmer amaranth biotype had approximately 33 to 111 relative copies of the *EPSPS* gene (Figure 3). These results are consistent with previously reported GR Palmer amaranth biotypes from Georgia and Mississippi with 33 to 100 *EPSPS* gene copies (Gaines et al. 2010; Ribeiro et al. 2014). In contrast, GR spiny amaranth (*Amaranthus spinosus* L.) from Mississippi and GR Italian ryegrass from Arkansas have been reported with 26 to 37 and 15 to 25 relative *EPSPS* gene copies, respectively (Nandula et al. 2014; Salas et al. 2012). Furthermore, lower folds of *EPSPS* gene amplification (2-fold to 10-fold) have been reported in GR Palmer amaranth biotypes from New Mexico; GR kochia biotypes from Colorado, Montana, and Kansas; and GR tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] biotypes (Kumar et al. 2015; Lorentz et al. 2014; Mohseni-Moghadam et al. 2013a; Wiersma et al. 2015).

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## Practical Implications

Results from this research suggest that plant height influences glyphosate resistance in GR Palmer amaranth and that the CT-Res Palmer amaranth biotype has evolved high-level (54-fold to 69-fold) resistance to glyphosate compared with the KS-Sus biotype. The molecular test further confirmed that the GR Palmer amaranth from Connecticut has evolved resistance to glyphosate via *EPSPS* gene amplification by 33-fold to 111-fold compared with the AL-Sus biotype. The current research project did not test any nontarget-based mechanisms (such as alteration in absorption, translocation, sequestration or metabolism) of glyphosate resistance in the CT-Res biotype; therefore, further research should determine whether additional mechanisms of resistance are involved. Nonetheless, the occurrence of GR Palmer amaranth in Connecticut is a serious concern, considering that glyphosate is the most common herbicide used for weed control. These results clearly suggest that effective alternative (other than glyphosate) PRE and POST herbicides (with multiple SOAs) would be needed to control this GR Palmer amaranth biotype. Field surveys are underway to collect more Palmer amaranth biotypes in Connecticut to assess the distribution of GR biotypes. Future studies will evaluate the response of GR Palmer amaranth biotype to alternative PRE and POST herbicides for various cropping systems in Connecticut.

In addition to effective herbicide programs, Connecticut producers should also consider adopting integrated Palmer amaranth control strategies, including cultural practices (such as cover crops, competitive crop rotations/sequences, optimum crop seeding rates and row spacing, etc.), mechanical practices (strategic tillage, electrocution, harvest weed control techniques, etc.), and precision agricultural technologies (drones for weed scouting, precision sprayers, etc.) for managing GR Palmer amaranth seedbanks and its further spread.

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