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Mechanism of Resistance of Evolved Glyphosate-Resistant Palmer Amaranth (*Amaranthus palmeri*)

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ABSTRACT: Evolved glyphosate resistance in weedy species represents a challenge for the continued success and utility of glyphosate-resistant crops. Glyphosate functions by inhibiting the plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). The resistance mechanism was determined in a population of glyphosate-resistant Palmer amaranth from Georgia (U.S.). Within this population, glyphosate resistance correlates with increases in (a) genomic copy number of *EPSPS*, (b) expression of the *EPSPS* transcript, (c) EPSPS protein level, and (d) EPSPS enzymatic activity. Dose response results from the resistant and an F_2 population suggest that between 30 and 50 *EPSPS* genomic copies are necessary to survive glyphosate rates between 0.5 and 1.0 kg ha^{-1}. These results further confirm the role of *EPSPS* gene amplification in conferring glyphosate resistance in this population of Palmer amaranth. Questions remain related to how the *EPSPS* amplification initially occurred and the occurrence of this mechanism in other Palmer amaranth populations and other glyphosate-resistant species.

KEYWORDS: gene amplification, herbicide resistance, transgenic crops, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), Palmer amaranth (*Amaranthus palmeri* S. Wats.)

INTRODUCTION

Evolution of resistance to glyphosate (*N*-[phosphonomethyl]glycine) in weedy species is a challenge for the continued success and utility of glyphosate-resistant (GR) crops.[1] One weed species originally well controlled with glyphosate in GR crops is Palmer amaranth (*Amaranthus palmeri* S. Wats.), a dioecious plant that is a major weed pest throughout the southeastern United States.[2] This species is described as resistance prone with evolved resistance to several different herbicides occurring in Palmer amaranth populations, including triazine, acetolactate-synthase inhibitor, and dinitroaniline herbicides.[3−8] Recently, glyphosate resistance was detected in Palmer amaranth populations in numerous U.S. states including Georgia,[9] Tennessee,[10] Arkansas,[11] and North Carolina, New Mexico, Alabama, Mississippi, Missouri, and South Carolina.[4]

Evolved glyphosate resistance in Palmer amaranth was first reported in 2006 in a population from the U.S. state of Georgia.[9] Glyphosate resistance in Palmer amaranth is now widespread.[12] The first Georgia Palmer amaranth population exhibited no changes in glyphosate uptake and translocation in comparison to a susceptible population,[9] and no known target-site mutations associated with glyphosate resistance were identified in the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene sequence.[13] However, DNA blots provided initial evidence to suggest *EPSPS* gene amplification was present in the resistant population. *EPSPS* gene amplification had not previously been identified as a glyphosate resistance mechanism in weeds, although the most common glyphosate resistance mechanism selected in experimental plant cell culture research has been increased EPSPS activity, typically due to gene amplification.[14]

In previously reported research, it was shown that within the Georgia population, glyphosate resistance correlated with increases in (a) genomic copy number of *EPSPS*, (b) expression of the *EPSPS* transcript, (c) EPSPS protein level, and (d) EPSPS enzymatic activity.[13] It appeared that amplification of the *EPSPS* gene produced an abundant supply of EPSPS able to act as a molecular sponge to absorb glyphosate, enabling uninhibited EPSPS to continue functioning following glyphosate treatment.[15] The extent of the gene amplification was variable in the resistant population, with a 40−>100-fold increase in *EPSPS* copy number measured by quantitative real-time PCR in Palmer amaranth plants from resistant relative to susceptible populations.[13] However, uncertainties concerning resistance due to gene amplification remain, such as how many *EPSPS* genomic copies are necessary to confer resistance to a typical glyphosate application rate. An additional question is the precise relationship between copy number and resistance level. For example, an interesting question is whether an individual with a 20-fold increase in *EPSPS* genomic copy number is as resistant as an individual with a 50- or 100-fold increase in *EPSPS* copies. Here, in this new research, we report additional evidence to support the hypothesis that glyphosate resistance level in Palmer amaranth increases with higher *EPSPS* genomic copy number and highlight questions regarding the *EPSPS* gene amplification that remain to be answered.

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MATERIALS AND METHODS

Plant Material. The glyphosate-resistant (R) Palmer amaranth population was collected from a field site in Macon County, Georgia,[9] and the glyphosate-susceptible (S) Palmer amaranth population was collected from the University of Georgia Ponder Farm Research Station. Both R and S plants were grown in a greenhouse and measured for glyphosate resistance using an in vivo leaf disk assay,[16] in which S plants accumulate shikimate at low glyphosate doses and R plants do not.[9,13] Shikimate accumulates in plants when EPSPS is inhibited by glyphosate because shikimate-3-phosphate, a substrate in the reaction catalyzed by EPSPS, converts to shikimate and accumulates more quickly than it can be consumed in other metabolic pathways.[17] An S × R F_1 half-sibling family was produced as described previously[13] by crossing three confirmed male R plants to a confirmed S female plant. Plants were shaken daily to ensure adequate cross-pollination, and upon maturity seeds were harvested and stored at 4 °C.

An F_2 population was produced as previously reported[13] by germinating seeds from the S × R F_1 family and spraying at the three-leaf stage with 0.4 kg ae ha^{-1} commercially formulated glyphosate (potassium salt, Roundup Weather Max, Monsanto Co., St. Louis, MO), a dose previously determined to be lethal to 100% of S but 0% of R plants. One surviving F_1 male was selected for crossing to one surviving F_1 female to generate an F_2 population (designated S × R/S × R). These two surviving F_1 individuals were isolated in a greenhouse prior to flowering, and their inflorescences were manually contacted daily to ensure cross-pollination. Seeds from the female plant were harvested and stored at 4 °C.

Shikimate Accumulation Assay. Three individuals from the S population, eight from the S × R F_1 family, and three from the R population were measured for shikimate accumulation using an in vivo leaf disk assay[16] in 10 mM ammonium phosphate buffer at three doses of 125, 250, and 500 μM glyphosate. A shikimate standard curve was used to quantify shikimate accumulation in the experimental samples.

EPSPS Genomic Copy Number. Six individuals from the S × R F_1 were grown in small pots, and leaves were collected from each plant for genomic DNA extraction and determination of genomic *EPSPS* copy number. In a separate experiment, 52 plants of the F_2 and 15 plants each of R and S were grown to the four-leaf stage as previously described.[13] One leaf from each plant was sampled for genomic DNA extraction and measurement of genomic EPSPS copy number.

Leaf tissue samples were immediately frozen in liquid nitrogen, ground in a 1.5 mL microcentrifuge tube, and stored at −80 °C. Genomic DNA was extracted using the Qiagen DNEasy Plant Mini Kit (Qiagen, Valencia, CA), quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and checked for quality by gel electrophoresis. DNA concentrations were adjusted to 1 ng μL^{-1} in sterile HPLC grade water.

Quantitative real-time PCR (qPCR) was used to measure EPSPS genomic copy number relative to acetolactate synthase (ALS) genomic copy number, and primer sets and qPCR conditions were as described previously.[13] Threshold cycles (Ct) for EPSPS and ALS were calculated using iCycler iQ v. 3.1 (Bio-Rad Laboratories, Hercules, CA). Data were analyzed using a modification of the $2^{-\Delta\Delta Ct}$ method[18] to express genomic copy number of EPSPS relative to ALS as ΔCt = (Ct, ALS − Ct, EPSPS), and relative increase in genomic EPSPS copy number was expressed as $2^{\Delta Ct}$. Each sample was run in triplicate to calculate the mean and standard error of the increase in EPSPS copy number relative to ALS. The *EPSPS* copy number data for the F_2, R, and S populations have been reported previously,[13] and the copy number data for the S × R F_1 family are reported here for the first time.

Glyphosate Dose Reponse. The R, S, and F_2 populations were assessed for response to glyphosate in a greenhouse experiment. Twenty seeds were planted on moistened commercial potting soil in 5 by 5 cm inserts, covered with 0.5 cm of additional soil, and placed in a 4 °C cold room for 7 days. The flats were transferred to germination chambers for two cycles of a temperature regimen that had been previously determined to stimulate rapid and simultaneous germination (data not shown): 18 °C for 6 h, 30 °C for 6 h, 42 °C for 6 h, and 30 °C for 6 h, along with 18 h of light. The flats with germinated seedlings were then placed in a greenhouse and fertilized with slow-release granular fertilizer. Seedlings were treated with glyphosate at the three-leaf stage. Glyphosate was applied in a pressurized spray chamber calibrated to deliver herbicide dissolved in 187 L of water ha^{-1} at 206 kPa. Plants were rated 15 days after treatment (DAT) for survival. Plants were considered to have survived if they had new growth from primary or secondary shoot meristems. A glyphosate dose response was conducted using 0, 0.08, 0.2, 0.33, 0.47, 0.99, 1.97, 3.94, and 6.3 kg ae glyphosate ha^{-1}. The experimental design consisted of three replications of each population at each dose, and the experiment was conducted three times.

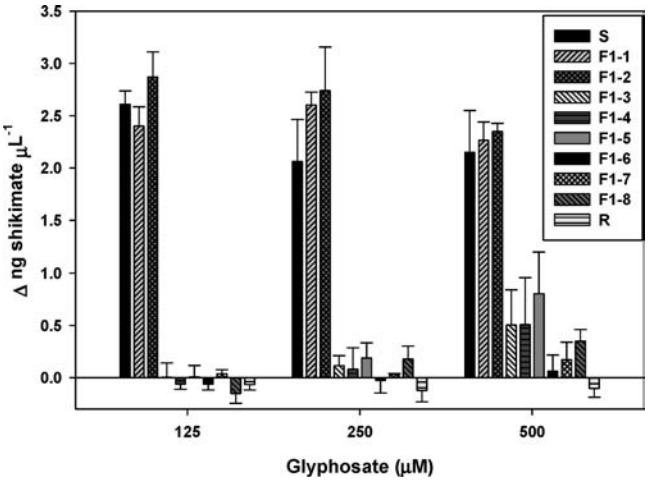

Figure 1. Variable levels of glyphosate resistance occur in progeny of crosses between glyphosate-susceptible (S) and glyphosate-resistant (R) Palmer amaranth: mean shikimate accumulation and standard errors in a leaf-disk assay using S, R, and eight F_1 (S × R) Palmer amaranth plants, relative to untreated leaf disks.

Dose response data from R and S populations were analyzed using logistic regression analysis, where x is log(dose g ae ha^{-1}), n is the number tested at each dose, Y is the number that survived at each dose (of n), and p is the true probability that an individual plant will live. Thus, Y is binomial (N,p) at each dose i, and the logistic model is

$$p_i = \frac{c + (d - c)\exp(\beta_0 + \beta_1 x_i)}{1 + \exp(\beta_0 + \beta_1 x_i)} \quad (1)$$

where β_0 is the intercept, β_1 is the slope, c is the lower limit, and d is the upper limit. Estimation was based on the maximum likelihood method in SAS PROC PROBIT.[19] The logistic model was used to estimate the dose required for 50% mortality (LD_{50}). Means and standard errors were calculated for dose response data from the R, S, and F_2 populations.

RESULTS AND DISCUSSION

Shikimate Accumulation. Plants from the S population accumulated shikimate at all three glyphosate doses, whereas plants from the R population did not accumulate shikimate at the highest dose tested (Figure 1). The eight plants from the S × R F_1 family did not exhibit a uniform shikimate response. Two individuals had a response similar to the S population, with shikimate accumulation at all doses tested (Figure 1). The other six F_1 individuals accumulated shikimate only at the highest dose tested (Figure 1).

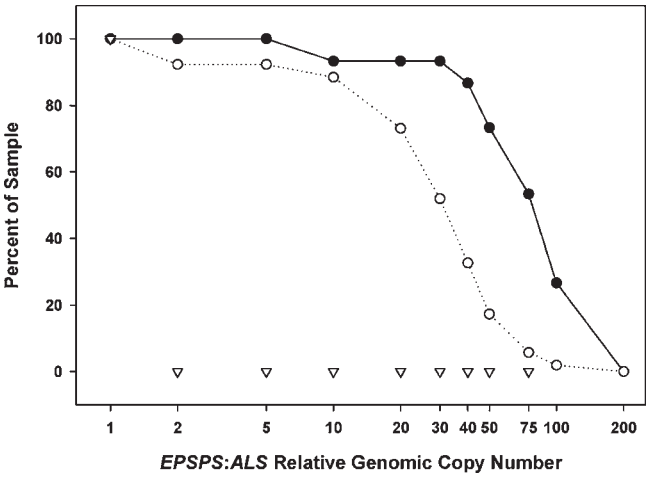

Figure 2. Palmer amaranth genomic copy number distribution of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) relative to acetolacte-synthase (*ALS*) in three populations: glyphosate-resistant (R, filled circles and solid line, n = 15), glyphosate-susceptible (S, open triangles, n = 15), and an F_2 (S × R/S × R) population (open circles and dotted line, n = 52). Data points indicate the percent of sampled plants with *EPSPS*:*ALS* relative genomic copy number equal to or higher than the corresponding value on the x-axis.

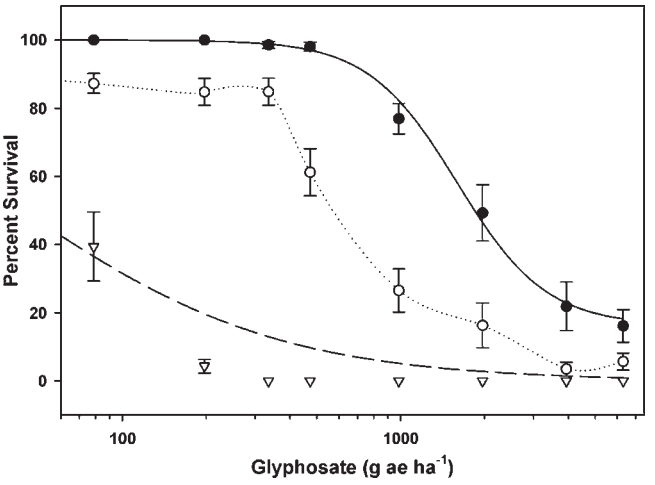

Figure 3. Survival of Palmer amaranth glyphosate-susceptible (S, open triangles and dashed line), glyphosate-resistant (R, filled circles and solid line), and F_2 (S × R/S × R, open circles and dotted line) in glyphosate dose response. Lines for R and S are binomial logistic regression (eq 1). Data points are means and standard errors.

EPSPS Genomic Copy Number. A range of *EPSPS* genomic copy numbers was observed in the six individuals from the S × R F_1 family, with genomic copy numbers relative to *ALS* of 1, 1, 16, 18, 21, and 39. It has previously been shown that the S population *EPSPS* genomic copy number was 1, and individuals in the R population had a range of copy number increases, with most individuals between 40- and 100-fold increase.[13] This S × R F_1 contained glyphosate-susceptible individuals and individuals with no increase in *EPSPS* genomic copy number. The F_1 also contains individuals with increases in *EPSPS* genomic copy number, generally less than what was previously observed in the R population, and it contains individuals with a lower glyphosate resistance level than the R population based on the shikimate assay. The relationship between *EPSPS* copy number and glyphosate resistance level in the F_1 requires additional work, and the stability of copy number transmission from parents to F_1 progeny remains to be clarified and its impact understood.

Previously reported[13] data from R, S, and F_2 individuals were graphed differently here to represent the population distributions of *EPSPS* genomic copy number, showing the percent of each population with copy number higher than selected values (Figure 2). All S individuals had an *EPSPS* relative copy number of 1. In the F_2, 50% of individuals had copy numbers of >30, whereas 50% of the individuals in the R population had copy numbers of >75 (Figure 2).

Glyphosate Dose Response. Dose response experiments showed clearly that the R population was glyphosate-resistant and the S population was susceptible (Figure 3), as expected on the basis of previous studies.[9] The LD_{50} determined for S was 0.04 kg ha^{-1}, and the LD_{50} for R was 1.6 kg ha^{-1}. The F_2 population dose response was intermediate between those of the R and S populations, containing both highly susceptible and highly resistant individuals and a range of intermediate phenotypes (Figure 3).

A comparison between copy number distribution in the F_2 and R populations (Figure 2) and the glyphosate dose response of these populations (Figure 3) provides additional support for the role of *EPSPS* gene amplification in conferring glyphosate resistance in this population of Palmer amaranth. For example, 80% of the R population had 50 or more *EPSPS* copies, and approximately 80% of the R population survived at a glyphosate rate of 0.99 kg ha^{-1}. Twenty percent of the F_2 had >50 *EPSPS* copies, and slightly more than 20% survived at a glyphosate rate of 0.99 kg ha^{-1}. Twenty percent of the R population had >100 *EPSPS* copies, and 20% of the population survived at a glyphosate rate of 3.94 kg ha^{-1}. For the F_2, 80% had >10 *EPSPS* copies, and 80% survived at 0.33 kg ha^{-1} (compared to 0% of the S population surviving at 0.33 kg ha^{-1}). Approximately 60% of the F_2 had >30 copies, and 60% survived at 0.47 kg ha^{-1}. These results strongly suggest that glyphosate resistance level increases as *EPSPS* genomic copy number increases. These results also suggest that between 30 and 50 *EPSPS* copies are necessary to survive rates between 0.47 and 0.99 kg ha^{-1}, which includes most typical field application rates for glyphosate.

A broad range of *EPSPS* genomic copy numbers was observed in an F_2 population (Figure 2). Fluorescent in situ hybridization has previously been used to observe *EPSPS* loci on all chromosomes visible in a chromosome spread derived from a glyphosate-resistant Palmer amaranth plant.[13] The dynamic nature of *EPSPS* copy number inheritance as well as the seemingly random insertion of *EPSPS* copies across Palmer amaranth chromosomes suggests amplification of this locus may be mediated by a mobile genetic element. The exact nature of *EPSPS* gene amplification transmission across generations and the original mechanism of amplification are uncertain. Additional information about the mechanism of amplification, the nature of copy number transmission across generations, and the occurrence of this mechanism in other Palmer amaranth populations and other glyphosate-resistant species is necessary to fully understand the novel mechanism of glyphosate resistance due to *EPSPS* gene amplification.

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■ ABBREVIATIONS USED

EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; ALS, acetolactate synthase; S, glyphosate-susceptible; R, glyphosate-resistant.

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