DISSERTATION

MOLECULAR GENETICS OF GLYPHOSATE RESISTANCE IN PALMER AMARANTH (*AMARANTHUS PALMERI* L.)

Submitted by

Todd A. Gaines

Department of Bioagricultural Sciences and Pest Management

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2009

COLORADO STATE UNIVERSITY

December 15, 2008

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TODD A. GAINES ENTITLED MOLECULAR GENETICS OF GLYPHOSATE RESISTANCE IN PALMER AMARANTH (*AMARANTHUS PALMERI* L.) BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Stephen Chisholm

Sarah Ward

Christopher Preston

Jan Leach, Co-Advisor

Philip Westra, Advisor

Tom Holtzer, Department Head

ABSTRACT OF DISSERTATION

MOLECULAR GENETICS OF GLYPHOSATE RESISTANCE IN PALMER AMARANTH (*AMARANTHUS PALMERI* L.)

Glyphosate resistant Palmer amaranth populations were identified in Georgia in 2004. Studies were undertaken to characterize inheritance, the molecular basis of resistance, and the potential for gene transfer to related *Amaranthus* species. Dose response results support rejecting a monogenic inheritance hypothesis in favor of an alternative polygenic, additive inheritance model. Apomixis in genetic populations used for inheritance studies is probably occurring and makes interpretation of inheritance difficult. Glyphosate resistance in Palmer amaranth appears to be incompletely dominant and may be polygenic. No target site mutations known to confer resistance were identified in resistant alleles of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, the target of glyphosate. Estimation of gene copy numbers of EPSPS relative to acetolactate synthase (ALS) in gDNA by quantitative PCR (qPCR) suggested that resistant plant genomes contain 64 to 128 times more copies of EPSPS than susceptible plants. qPCR on cDNA revealed that EPSPS was expressed approximately 35 times higher in resistant plants. Elevated EPSPS copy number is heritable and correlates with expression level and resistance in F_2 populations. The molecular basis of resistance is likely due to increased production of EPSPS due to gene amplification. This is the first documented occurrence of EPSPS gene amplification in a weed population

under glyphosate selection pressure. The risk of resistance gene transfer was measured with field studies and hand crosses with *A. hybridus*, *A. retroflexus*, *A. powellii*, *A. spinosus*, and *A. tuberculatus*. Glyphosate application (0.4 kg ha^{-1}) was used to screen for resistant progeny from the crosses. Hybridization with *A. spinosus* occurred in both years of the field study and in hand crosses, with average frequency ranging from <0.01% to 1.4%. Hybrids with *A. spinosus* were either monoecious or dioecious. Monoecious plants produced seed through self-pollination, and the F_2 progeny were segregating for resistance. Hybridization occurred in the 2007 field study with *A. hybridus* (<0.01%) and *A. tuberculatus* (0.08% and 0.19% for two accessions), all of the hybrid plants were dioecious, and none produced seed. The highest risk for glyphosate resistance gene transfer from *A. palmeri* is to *A. spinosus*.

Todd A. Gaines
Department of Bioagricultural Sciences and Pest Management
Colorado State University
Fort Collins, Colorado 80523
Spring 2009

ACKNOWLEDGEMENTS

Funding for this dissertation was generously provided by Monsanto Company, including Jeff Tichota, Paul Isakson, Jennifer Ralston, and Doug Sammons. I would like to thank my committee, Stephen Chisholm, Sarah Ward, and Christopher Preston, and co-advisor, Jan Leach, for their advice and encouragement. I would especially like to thank my advisor, Phil Westra, for his support and guidance. I am grateful to everyone at the University of Georgia who helped get this project started. I owe a great deal of debt to everyone in the Leach lab and the weeds lab who taught me lab techniques, helped me out, gave me good advice, and have been great friends, including Jacob Snelling, Rebecca Davidson, Seweon Lee, Hiromichi Ishihara, Myron Bruce, Gena Diaz, Bekir Bukun, Melissa Bridges, Joe Vassios, and Galen Brunk. Finally, many thanks to my wife Jerica Rasmussen for tolerating someone who studies weeds every day but won't do anything about the ones in the back yard – all my love.

TABLE OF CONTENTS

CHAPTER 1: INHERITANCE

Inheritance of glyphosate resistance in Palmer amaranth (*Amaranthus palmeri* L.)

Todd A. Gaines, Sarah Ward, Christopher Preston, Bekir Bukun, and Philip Westra.

ABSTRACT

Glyphosate resistance in Georgia Palmer amaranth populations is a major agronomic concern. Inheritance of the resistance trait was examined by making reciprocal crosses between resistant (R) and susceptible (S) populations. Both F_1 crosses were backcrossed (BC) to a susceptible maternal parent (S//R/S and S//S/R) and half-siblings from the S/R F_1 were crossed to generate an F_2 population. All seven populations were used for glyphosate dose response studies to test a monogenic inheritance hypothesis. Resistance was inherited from both maternal and paternal sources. Both the S/R and R/S F_1 populations had dose responses (LD_{50} of 0.21 and 1.07 kg ha^{-1}, respectively) more similar to their maternal parent (LD_{50} of 1.6 and 0.04 kg ha^{-1} for R and S). Both BC and the F_2 population had significantly higher survival at lower rates than expected survivals based on a monogenic hypothesis. Expected frequencies were calculated based on F_1 and parental dose responses. Dose response of multiple F_1 populations indicated that not all individuals in the F_1 populations used to generate BC and F_2 populations were heterozygous. The dose response results would support rejecting a monogenic hypothesis in favor of an alternative polygenic, additive inheritance model. One explanation for heterogeneity in both F_1 populations is apomixis. Because the level of apomixis is unknown, expected survival rates could not be adjusted to account for apomixis. Glyphosate resistance in Palmer amaranth is nuclear inherited, appears to be incompletely dominant and may be polygenic.

Evolution of resistance to glyphosate (N-[phosphonomethyl] glycine) in weedy species is a risk to the continued success of transgenic glyphosate resistant crops (Powles 2008). Glyphosate resistant weeds pose management challenges and may require the use of alternative biotechnology-based weed management strategies (Behrens et al. 2007). Palmer amaranth is a troublesome agronomic weed, in part because of multiple herbicide resistance traits (Vencill et al. 2008). Glyphosate resistance has recently been reported in Palmer amaranth populations in Georgia (Culpepper et al. 2006), Tennessee (Steckel et al. 2008), North Carolina (Heap 2008), and Arkansas (Norsworthy et al. 2008). The resistance mechanism in the Georgia population was shown to not be due to whole plant uptake and translocation (Culpepper et al. 2006) and the authors suggested that the mechanism may be target-site based.

The inheritance of a herbicide resistance trait is one factor influencing the evolution of herbicide resistance (Diggle and Neve 2001). The inheritance of glyphosate resistance has been studied in several weed species. Rigid ryegrass (*Lolium rigidum*) from California had a high proportion of intermediate resistant plants in an F_2 relative to resistant and susceptible phenotypes (1:14:1), and the authors concluded that the resistance was controlled by at least two genes (Simarmata et al. 2005). Inheritance of glyphosate resistance in horseweed (*Conyza canadensis*) was found to be due to an incompletely dominant single gene (Zelaya et al. 2004). Resistance in goosegrass (*Eleusine indica*) was also found to be an incompletely dominant, nuclear inherited single

gene (Ng et al. 2004). Resistance in rigid ryegrass from Australia was found to be a single, incompletely dominant nuclear inherited gene (Lorraine-Colwill et al. 2001). The level of glyphosate resistance in Palmer amaranth is 6 to 8 fold (Culpepper et al. 2006). The objective of this study was to test models of glyphosate resistance in Palmer amaranth for maternal inheritance, number of genes, and level of dominance determine the inheritance of by testing models of maternal inheritance, monogenic and dominant.

MATERIALS AND METHODS

Plant Material

Seeds of glyphosate resistant Palmer amaranth were collected from a field site in Macon County, Georgia (Culpepper et al. 2006). Seeds of glyphosate susceptible Palmer amaranth were collected from a different field site in Georgia. Seeds of resistant and susceptible were germinated, transplanted into large pots and moved into a greenhouse under natural light conditions supplemented with 400 W sodium halide lamps to provide a 14-h daylength. Daytime temperatures were approximately 24 C and nighttime temperatures were approximately 18 C. Resistant plants were confirmed using the in-vivo leaf disc assay of Shaner et al. (2005). Palmer amaranth is dioecious, with male and female flowers on separate plants (Murray 1940). Plants were covered with pollination bags prior to flowering initiation. When flowers were visible, susceptible males were moved into a different greenhouse. Resistant females were placed in the greenhouse with susceptible males, and resistant males were placed next to susceptible females in a

separate greenhouse to create reciprocal F_1 populations. Plants were shaken daily to ensure adequate pollination.

Seeds from the female plants were harvested by hand, labeled, and stored at 4 C for two months. Seeds from the R/S F_1 and S/R F_1 were germinated and grown to flowering. The S/R F_1 was sprayed with 0.4 kg ae ha^{-1} commercially formulated glyphosate at the 3-leaf stage to ensure that plants used for crossing had inherited and were heterozygous for the resistance trait, as apomixis may occur in Palmer amaranth (Trucco et al. 2007). The R/S F_1 plants were verified using an in-vivo leaf disc assay (Shaner et al. 2005). Only one male from each F_1 was selected for backcrossing to susceptible females. F_1 males were placed in separate greenhouses. One female from the S/R F_1 was placed next to an S/R F_1 male to generate an F_2 population through half-sibling mating. Seeds from the female plants were harvested by hand, labeled, and stored at 4 C for two months.

Glyphosate Dose Response

Palmer amaranth populations were screened for glyphosate resistance across a range of nine doses, including 0, 0.08, 0.2, 0.33, 0.47, 0.99, 1.97, 3.94, and 6.3 kg ae ha^{-1}. Populations screened included the field-collected resistant (R), susceptible (S), R/S F_1, S/R F_1, backcross of each F_1 to S, and the F_2 from mating S/R F_1 half-siblings. The experimental design consisted of three replications of each population at each dose, and the experiment was conducted three times. Seeds were planted on moistened commercial potting soil in 5 by 5 cm inserts, covered with a thin layer of additional soil, and placed in a 4 C cold room for 7 d. The flats were transferred to germination chambers for two cycles of the following temperate regime: 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 light. Germinated seedlings were then placed in a greenhouse under the previously described conditions and fertilized with slow-release granular fertilizer (Osmocote, Scotts Company). Seedlings were treated with glyphosate at the 3-leaf stage. Glyphosate was applied in a pressurized spray chamber calibrated to deliver 187 L ha^{-1} at 206 kPa. Plants were rated 15 d after treatment (DAT) for survival. Plants were considered to have survived if they were actively growing.

Data Analysis

Dose response data 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 (out 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:

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

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 (SAS 2004). Chi-square statistics were calculated to determine the goodness-of-fit of the regression lines. The logistic model was used to estimate the slope, LD_{50}, and 95% fiducial limits for resistant, susceptible, and the reciprocal F_1 populations.

The hypothesis of monogenic inheritance was tested by analyzing data from the backcross and F_2 generations at each dose using 2×2 contingency analysis and Fisher's exact test (Tabashnik 1991). The proportion surviving at each dose for the resistant, susceptible, and the reciprocal F_1 populations was used to calculate the expected survival for each backcross and the F_2 population under a monogenic hypothesis as described by Tabashnik (1991):

$$BC\text{: } \hat{p}_x = 0.5(W_{SR} + W_{SS})$$

$$F_2\text{: } \hat{p}_x = 0.25W_{RR} + 0.5W_{SR} + 0.25W_{SS}$$

where $\hat{p}$ is expected proportion alive at dose x, W_{SR}, W_{SS}, and W_{RR} are the F_1, susceptible, and resistant parent observed proportion alive at dose x, respectively. Expected proportion alive at dose x ($\hat{p}$) was used to calculate the expected number alive (Y) at dose x using n, the total number of plants exposed to dose x, as $Y_x = \hat{p}n$.

Screening Multiple F_1 Populations

Progeny from multiple females used for the S/R (3) and R/S (4) F_1 crosses, along with resistant and susceptible plants, were tested at two doses, 0.2 and 0.99 kg ae ha^{-1}, with three replications. Plant growth conditions were as described previously. The experiment was repeated twice. The proportion of surviving plants was recorded and analyzed with ANOVA in SAS PROC GLM (SAS 2004) to determine whether survival was different among the various F_1 populations at the two doses. Means were compared using an F-protected LSD at α=0.05.

RESULTS AND DISCUSSION

The LD_{50} estimates from the logistic dose response model were 1.6 kg ha^{-1} for the resistant population and 0.04 kg ha^{-1} for the susceptible population (Table 1 - 1). These estimates are similar to the greenhouse-based estimates (1.2 and 0.15 kg ha^{-1},

respectively) of Culpepper et al. (2006). The estimated LD_{50} for each F_1 population was closer to its maternal parent than the midpoint, with the R/S F1 having an LD_{50} of 1.07 kg ha^{-1}, and the S/R F1 having an LD_{50} of 0.21 kg ha^{-1} (Table 1 - 1). The logistic dose response of each F_1 population falls closer to each maternal parent than the midpoint (Figure 1 - 1).

Observed survival across the dose range in the BC and F_2 populations shows a rapid drop in survival from 100% at low rates, a decreasing slope in the middle of the rate range, and survival at 1.97 kg ha^{-1} only in the F_2 population (Figure 1 - 2). These results could be consistent with a monogenic hypothesis, showing elimination of SS genotypes at low rates, elimination of RS genotypes at middle rates, and survival of RR genotypes in the F_2 at high rates. However, tests for monogenic inheritance in both BC and the F2 populations showed significantly higher survival than expected at rates of 0.47 kg ha^{-1} and less (Table 1 - 2). The S//S/R BC had significantly higher survival than expected at rates of 1.97 kg ha^{-1} and less, and the S//R/S BC had less survival than expected at 1.97 kg ha^{-1} (Table 1 - 2). Using the model of Tabashnik (1991), the progeny in both backcross populations had higher survival rates at moderately low doses and one backcross had lower survival rates at moderately high doses, indicating that that the hypothesis of monogenic inheritance should be rejected in favor of an alternative polygenic, additive inheritance model.

Expected survival frequencies were based on survival rates at each dose in the R/S and S/R F_1 populations. The hypothesis of monogenic inheritance assumes that all individuals in an F_1 population are heterozygous (Tabashnik 1991). Multiple F_1 populations tested at two rates showed significant variation in survival (Table 1 - 3). The

S/R F_1 used for BC and F_2 populations had significantly less survival than two other S/R F_1 populations, and the R/S F_1 used for a BC population had significantly higher survival than one other R/S F_1 (Table 1 - 3). One R/S F_1 population had similar survival at 0.99 kg ha^{-1} as the field resistant population. These results indicate that not all individuals in the F_1 populations are heterozygous, and that expected survival frequencies based on F_1 dose response data are likely not estimating the heterozygous RS response accurately. It is also possible that the resistant population was not homogeneous for magnitude of resistance and the resistance inherited in the S/R F_1 was less dominant than the resistance in the R/S F_1. However, the S/R//S/R F_2 has a similar dose response in the presumably heterozygous response region as the S//R/S backcross, so this seems unlikely.

One possible explanation for heterogeneity in the F_1 populations is apomixis (Trucco et al. 2007), the production of individuals with the maternal homozygous genotype through asexual seed production. The variation between F_1 population responses and the similarity of the S/R and R/S F_1 populations to their maternal populations could both be explained if the proportion of apomicts varies between F_1 populations. If the level of apomixis were known, then an adjustment could be made to expected survival rates for the heterozygous genotype. Because the true level of apomixis in each cross is unknown, no adjustment for apomixis was made. Thus it cannot be concluded whether inheritance is monogenic. However, resistance is inherited both maternally and paternally, indicating that the trait is nuclear inherited, and an F_2 population generated from an S/R F_1 (paternal resistance source) segregated for resistance in a manner suggesting three genotypes were present (SS, RS, and SS) (Figure 1 - 2).

If model assumptions regarding heterozygosity of the F_1 population are valid, then the Tabashnik (1991) model would support polygenic, additive gene action. However, as F_1 populations are segregating due to the likely occurrence of apomixis, it is very difficult to estimate the heterozygous response. Thus it is difficult to precisely determine the number of genetic factors involved because the expected frequencies are biased by the observed segregating dose response of the F_1 populations.

Glyphosate resistance in Palmer amaranth is nuclear inherited. Heterozygous individuals are less resistant than homozygous but more resistant than the mid-point between the resistant and susceptible dose response lines, so resistance is incompletely dominant. The F_2 population is segregating for resistance but highly resistant and highly susceptible individuals are present at about 15% each. This could suggest that the resistance trait is polygenic, or it could indicate that the F2 population contains an over-abundance of heterozygous individuals due to the production of apomicts carrying the maternal (heterozygous) genotype. The over-abundance of intermediate resistant phenotypes in rigid ryegrass from California (Simarmata et al. 2005) is similar to these observations in Palmer amaranth, as well as resistance in rigid ryegrass being nuclear inherited and incompletely dominant, possibly indicating a similar mechanism is present in these biotypes of these two species.

REFERENCES

Behrens, M. R., N. Mutlu, S. Chakraborty, R. Dumitru, W. Z. Jiang, B. J. LaVallee, P. L. Herman, T. E. Clemente, and D. P. Weeks. 2007. Dicamba resistance: Enlarging and preserving biotechnology-based weed management strategies. Science 316:1185-1188.

Culpepper, A. S., T. L. Grey, W. K. Vencill, J. M. Kichler, T. M. Webster, S. M. Brown, A. C. York, J. W. Davis, and W. W. Hanna. 2006. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci. 54:620-626.

Diggle, A. J. and P. Neve. 2001. The population dynamics and genetics of herbicide resistance - A modeling approach, *In* S. B. Powles and D. L. Shaner, eds. Herbicide Resistance and World Grains. CRC Press, Boca Raton, FL.

Heap, I. 2008. The International Survey of Herbicide Resistant Weeds. Online. Available at www.weedscience.com (accessed October 21, 2008).

Lorraine-Colwill, D. F., S. B. Powles, T. R. Hawkes, and C. Preston. 2001. Inheritance of evolved glyphosate resistance in *Lolium rigidum* (Gaud.). Theor. Appl. Gen. 102:545-550.

Murray, M. J. 1940. The genetics of sex determination in the family *Amaranthaceae.* Genetics 25:409-431.

Ng, C. H., W. Ratnam, S. Surif, and B. S. Ismail. 2004. Inheritance of glyphosate resistance in goosegrass (*Eleusine indica*). Weed Sci. 52:564-570.

Norsworthy, J. K., G. M. Griffith, R. C. Scott, K. L. Smith, and L. R. Oliver. 2008. Confirmation and control of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in Arkansas. Weed Technol. 22:108-113.

Powles, S. B. 2008. Evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Mgmt. Sci. 64:360-365.

SAS Institute Inc. 2004. SAS OnlineDoc® 9.1.3. SAS Institute Inc., Cary, NC.

Shaner, D. L., T. Nadler-Hassar, W. B. Henry, and C. H. Koger. 2005. A rapid in vivo shikimate accumulation assay with excised leaf discs. Weed Sci. 53:769-774.

Simarmata, M., S. Bughrara, and D. Penner. 2005. Inheritance of glyphosate resistance in rigid ryegrass (*Lolium rigidum*) from California. Weed Sci. 53:615-619.

Steckel, L. E., C. L. Main, A. T. Ellis, and T. C. Mueller. 2008. Palmer amaranth (*Amaranthus palmeri*) in Tennessee has low level glyphosate resistance. Weed Technol. 22:119-123.

Tabashnik, B. E. 1991. Determining the mode of inheritance of pesticide resistance with backcross experiments. J. Econ. Entom. 84:703-712.

Trucco, F., D. Zheng, A. J. Woodyard, J. R. Walter, T. C. Tatum, A. L. Rayburn, and P. J. Tranel. 2007. Nonhybrid progeny from crosses of dioecious amaranths: Implications for gene-flow research. Weed Sci. 55:119-122.

Vencill, W. K., T. L. Grey, A. S. Culpepper, T. A. Gaines, and P. Westra. 2008. Herbicide-resistance in the Amaranthaceae. J. Plant Dis. Protect. Special Issue XXI:41-44.

Zelaya, I. A., M. D. K. Owen, and M. J. VanGessel. 2004. Inheritance of evolved glyphosate resistance in *Conyza canadensis* (L.) Cronq. Theor. Appl. Gen. 110:58-70.

TABLES

Table 1 - 1. Response to glyphosate of Palmer amaranth: susceptible (S), field-collected resistant (R), and their reciprocal F_1 progenies. Plants were treated with glyphosate at the 3-leaf stage.

Population	*n*	Slope ± SE	LD_{50} (95% FL) (kg ae ha^{-1})	df	χ^2
S	1,297	-2.15 ± 0.14	0.04 (0.03-0.06)	1	223.22****
F_1 (R/S)	1,416	-5.77 ± 0.49	1.07 (0.95-1.23)	1	140.73****
F_1 (S/R)	1,250	-2.67 ± 0.17	0.21 (0.18-0.25)	1	236.42****
R	1,674	-6.26 ± 0.60	1.60 (1.41-1.87)	1	109.51****

**** Significant ($P < 0.0001$).

Table 1 - 2. Contingency analysis using Fisher's exact test of the glyphosate response of backcross and F_2 progeny from parental glyphosate resistant (R) and susceptible (S) Palmer amaranth populations. Expected responses were calculated under the hypothesis of monogenic inheritance.

	----------Backcross: S//R/S----------				----------Backcross: S//S/R----------				----------F_2: S/R//S/R----------			
Glyphosate (kg ae ha^{-1})	No. tested	Observed survivors	Expected survivors	χ^2	No. tested	Observed survivors	Expected survivors	χ^2	No. tested	Observed survivors	Expected survivors	χ^2
0.00	238	238	237	1.00	346	346	344	2.01	184	184	184	0.00
0.08	174	172	120	57.54****	360	321	212	85.83****	215	184	159	9.01**
0.20	210	198	108	97.52****	301	217	49	190.11****	113	97	45	51.23****
0.33	221	182	107	56.23****	280	182	38	155.24****	175	155	67	95.38****
0.47	210	127	84	17.61****	301	148	32	106.64****	190	118	67	27.40****
0.99	235	86	70	2.46	303	58	25	15.20****	203	49	56	0.63
1.97	223	10	33	13.62***	285	16	2	11.24***	232	27	30	0.18
3.94	243	10	11	0.05	280	0	0	0.00	214	8	12	0.84
6.30	167	1	5	2.72	275	0	0	0.00	143	5	6	0.09

*, **, ***, **** - Significant at $P < 0.05$, < 0.01, < 0.001, and < 0.0001, respectively.

Table 1 - 3. Variation in observed glyphosate dose response of Palmer amaranth F_1 populations from crosses with either resistant (R) or susceptible (S) as maternal parent. Populations R/S 1 and S/R 3 were used to construct backcross and F_2 populations. Means within a column with the same letter are not significantly different ($P < 0.05$).

	% Survival			
	----Rate (kg ae ha^{-1})----			
Population	0.2		0.99	
S	0.26	A	0.00	A
R/S 1	0.98	E	0.65	EF
R/S 2	0.71	CD	0.26	BC
R/S 3	1.00	E	0.68	FG
R/S 4	0.99	E	0.54	DE
S/R 1	0.80	DE	0.35	C
S/R 3	0.46	B	0.18	AB
S/R 5	0.68	C	0.47	D
R	0.98	E	0.79	G

FIGURES

Figure 1 - 1. Observed glyphosate dose response of Palmer amaranth field-collected glyphosate resistant (R), susceptible (S), and their reciprocal F_1 crosses. Lines are binomial logistic regression and data points are means and standard errors.

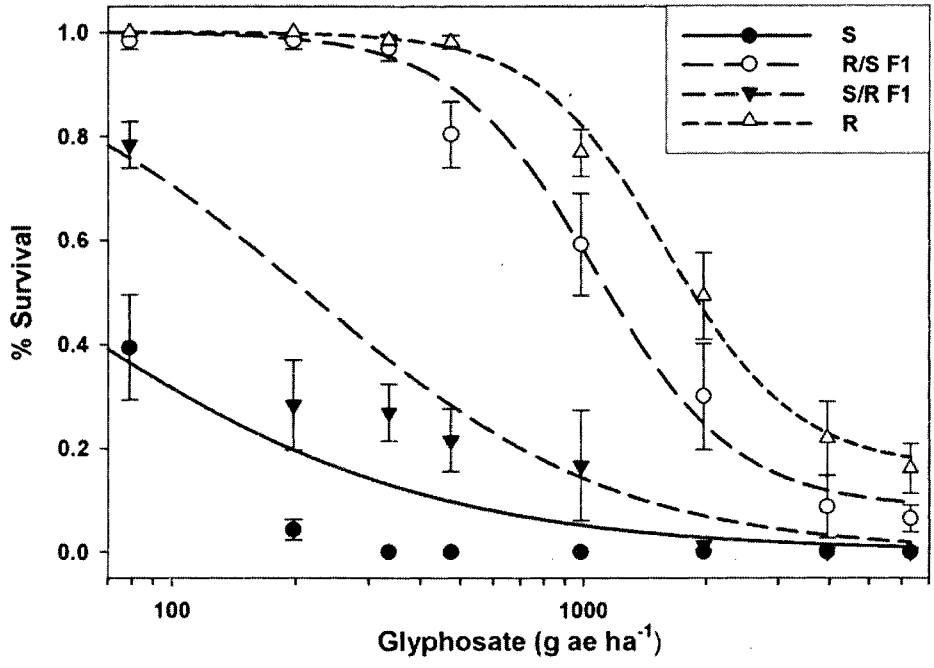
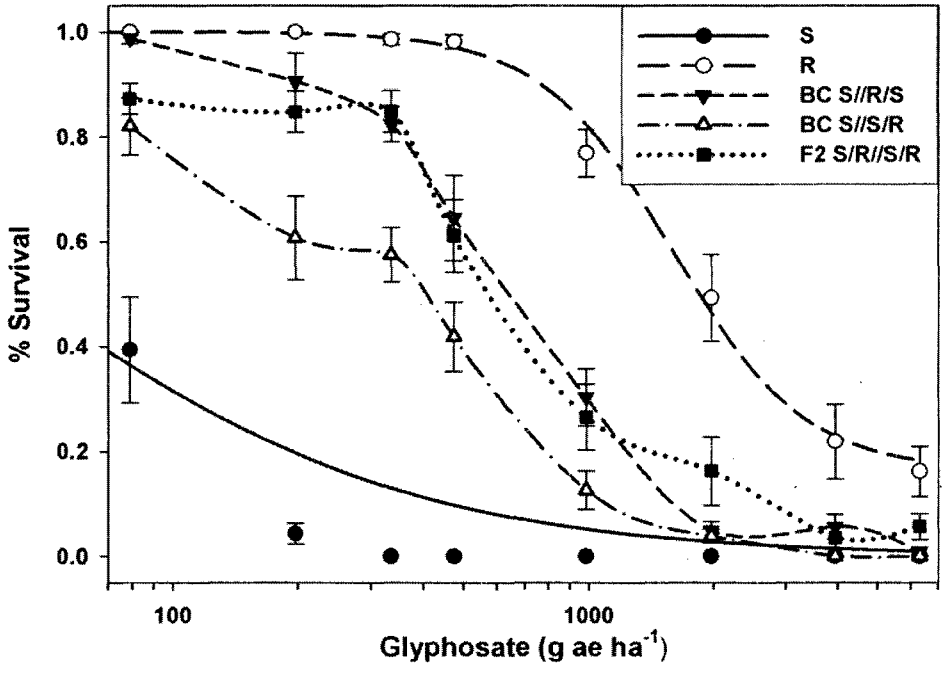


Figure 1 - 2. Observed glyphosate dose response data of Palmer amaranth susceptible (S), field-collected glyphosate resistant (R), backcross of R/S F_1 to S female, backcross of S/R F_1 to S female, and F_2 from mating S/R F_1 half-siblings. Lines for R and S are binomial logistic regression. Data points are means and standard errors.



CHAPTER 2: MOLECULAR BASIS OF GLYPHOSATE RESISTANCE

Molecular genetics of glyphosate resistance in Palmer amaranth (*Amaranthus palmeri* L.)

Todd A. Gaines, Christopher Preston, Philip Westra, Jan E. Leach, Stephen T. Chisholm, Dale L. Shaner, Scott J. Nissen, Bekir Bukun, Patrick J. Tranel, A. Stanley Culpepper, Timothy L. Grey, Theodore M. Webster, and William K. Vencill

ABSTRACT

Glyphosate resistant Palmer amaranth populations are being detected throughout the U.S. with increasing frequency. The molecular basis of resistance is unknown. Analysis of resistant and susceptible populations of Palmer amaranth from Georgia revealed that genomes of resistant plants contain more copies of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, the target of glyphosate, than susceptible plants. Estimation of gene copy numbers of EPSPS relative to acetolactate synthase (ALS) in gDNA by quantitative PCR (qPCR) revealed the same threshold cycle (Ct) for ALS and EPSPS in gDNA from susceptible plants and the same Ct for ALS in both resistant and susceptible plant gDNA. In contrast, the Ct for EPSPS in gDNA from resistant plants was six to seven cycles earlier than the Ct for ALS, suggesting that resistant plant genomes contain 64 to 128 times more copies of EPSPS than ALS. q-PCR on cDNA revealed that EPSPS was expressed at 30 to 40 times higher levels in resistant plants. Elevated EPSPS copy number is heritable and correlates with expression level and resistance in F_2 populations. Because no target site mutations known to confer resistance were identified in resistant alleles of the EPSPS gene, we hypothesize that the molecular basis of resistance is due to increased production of EPSPS due to gene amplification. However, the possibility exists that one or a few genomic copies have a target-site mutation that has not been detected. This is the first documented occurrence of EPSPS gene amplification in a glyphosate resistant weed population.

Evolution of resistance to glyphosate (N-[phosphonomethyl] glycine) in weedy species is a risk to the continued success of transgenic glyphosate resistant crops (Powles 2008). Glyphosate resistant weeds pose management challenges and produce an incentive for the development of alternative biotechnology-based weed management strategies (Behrens et al. 2007). Palmer amaranth is a troublesome agronomic weed, in part because of multiple herbicide resistance to photosystem II inhibitors, acetolactate synthase (ALS) inhibitors, and dinitroaniline herbicides (Vencill et al. 2008). Glyphosate resistance has recently been reported in Palmer amaranth populations in Georgia (Culpepper et al. 2006), Tennessee (Steckel et al. 2008), North Carolina (Heap 2008), and Arkansas (Norsworthy et al. 2008). The resistance mechanism in the Georgia population was not be due to whole plant uptake and translocation (Culpepper et al. 2006) and was proposed to be due to a target-site based mechanism.

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (E.C. 2.5.1.19) is in the shikimate pathway (Herrmann and Weaver 1999) and is the target of the widely used non-selective herbicide glyphosate (Steinrucken and Amrhein 1980). The shikimate pathway produces chorismate, which is used in the synthesis of the aromatic amino acids tyrosine, tryptophan, and phenylalanine, along with other phenolic compounds (Schmid and Amrhein 1995). The reaction catalyzed by EPSPS is the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to shikimate-3-

phosphate (S3P) (Padgette et al. 1996). Glyphosate is competitive with respect to PEP (Steinrucken and Amrhein 1980).

Resistance to glyphosate in plants can be conferred by expression of glyphosate-insensitive plant EPSPS due to one or more target-site mutations in EPSPS, expression of glyphosate-insensitive EPSPS from bacteria, over-expression of sensitive EPSPS, reduced glyphosate translocation, or enhanced glyphosate metabolism (Pline-Srnic 2006; Powles and Preston 2006; Sammons et al. 2007). Because of the particular mechanism of action of glyphosate and its usage patterns, Bradshaw et al. (1997) considered the evolution of glyphosate resistance in weeds unlikely to occur by metabolism, EPSPS over-expression, or EPSPS target-site mutation.

Target site mutations that provide varying levels of resistance to glyphosate and have been characterized for reaction efficiency (using the maize EPSPS numbering system) include Gly101Ala, Pro106Ser, and a combination of Thr102Ile and Pro106Ser (TIPS) (Dill 2005). Changes to EPSPS structure that decrease glyphosate binding generally reduce efficiency for binding PEP (Dill 2005). The Pro106Ser mutation has been found in glyphosate resistant weed species including goosegrass (*Eleusine indica*) (Baerson et al. 2002a; Kaundun et al. 2008; Ng et al. 2004). Moderate levels of resistance provided by the Pro106 mutation are sufficient for weeds to survive typical application rates (Powles and Preston 2006).

Altered glyphosate translocation from source leaves to the meristems has been documented as a resistance mechanism in rigid ryegrass (*Lolium rigidum*) and horseweed (*Conyza canadensis*) (Powles and Preston 2006). Investigation of glyphosate resistant horseweed showed reduced translocation out of treated leaves and into roots compared to

susceptible biotypes (Feng et al. 2004), and the authors suggested that the altered translocation was due to differences in cellular distribution of glyphosate and subsequent phloem loading and translocation.

Baerson et al. (2002b) found elevated EPSPS expression (2.5 to 3 times higher) in glyphosate resistant rigid ryegrass. The EPSPS extracted from resistant and susceptible plants was equally sensitive to glyphosate, but the enzyme activity was higher in resistant lines. The authors concluded that higher EPSPS mRNA expression was resulting in higher EPSPS production, but they were uncertain whether the magnitude of over-expression was sufficient to explain the observed resistance level. The glyphosate resistant lines accumulated shikimate after treatment, but less than susceptible lines. Baerson et al. (2002b) found that EPSPS is likely a gene family of two to five members in rigid ryegrass, but there was an apparent lack of EPSPS gene amplification in glyphosate resistant lines, even though the lines had 2.5 to 3 times higher EPSPS expression.

Shah et al. (1986) found that glyphosate resistance in a petunia cell line under glyphosate selection was due to 20-fold amplification of the EPSPS gene. DNA blots showed much stronger hybridization at one locus and at least one non-amplified locus. An EPSPS cDNA was cloned from the glyphosate resistant line and transformed into petunia cells. Regenerated petunia plants containing the EPSPS cDNA from the glyphosate resistant cell line fused with the CaMV 35S promoter survived a glyphosate dose higher than normal field use rates (0.9 kg ha^{-1}). EPSPS mRNA expression was estimated to be about 20 times higher in the regenerated line, using RNA blot dilutions.

Amplified EPSPS loci have been identified in glyphosate-resistant cell lines (Boerboom et al. 1990; Jones et al. 1996; Shah et al. 1986; Suh et al. 1993; Widholm et al. 2001). A review of glyphosate selection cell culture studies found that the most common glyphosate resistance mechanism selected in cell culture is increased EPSPS activity, typically due to gene amplification (Pline-Srnic 2006). Some tobacco cell lines maintained increased gene copy number, mRNA expression, and EPSPS production following removal of glyphosate selection (Jones et al. 1996).

The combination of expressing both sensitive and insensitive enzyme forms can confer herbicide resistance and adequate enzyme function in the absence of the herbicide. A maize cell line with an insensitive form of EPSPS but not increased EPSPS activity was isolated (Forlani et al. 1992). The cell line had an insensitive form with greatly reduced affinity for PEP and sensitive EPSPS with normal substrate affinity. Selection with sulfonylurea herbicide in tobacco cell culture resulted in amplification of the ALS gene and one copy had a point mutation for resistance (Harms et al. 1992). The ALS resistance was due to the copy with the point mutation. Over-expressing the TIPS EPSPS mutant (Sidhu et al. 2000) resulted in highly tolerant corn. Expression of resistant EPSPS in both vegetative and reproductive tissues is critical for glyphosate resistance. Cotton with expressing glyphosate-insensitive CP4 EPSPS behind the figwort mosaic virus (FMV) 35S promoter had male sterility, but adding an additional CP4 EPSPS behind the Arabidopsis *ef-1* promoter to the cassette improved expression in developing male gametes and conferred resistance at later application stages (Chen et al. 2006).

Glyphosate resistant Palmer amaranth is a serious agronomic problem affecting the southeastern U.S. An understanding of the molecular basis of the resistance

mechanism is needed. Resistance is not due to altered glyphosate uptake and translocation (Culpepper et al. 2006). As reviewed, glyphosate selection in cell culture can result in EPSPS gene amplification. The objective of this study was to determine whether glyphosate resistance in Palmer amaranth is caused by an EPSPS target-site mutation or by sensitive EPSPS over-expression due to gene amplification.

MATERIALS AND METHODS

Plant Material and Genetic Populations

Seeds of glyphosate-resistant Palmer amaranth were collected from a field site in Macon County, Georgia (Culpepper et al. 2006). Seeds of glyphosate susceptible Palmer amaranth were collected from a different field site in Georgia. Seeds of resistant and susceptible were germinated and transplanted into large pots. The resistance phenotype of each plant was confirmed using the in-vivo leaf disc assay of Shaner et al. (2005). Each plant was covered with pollination bags prior to flowering initiation. Resistant males were placed next to susceptible females to create an F_1 generation (S/R). Plants were shaken daily to ensure adequate pollination.

Seeds from the susceptible female plants were harvested by hand, labeled, stored at 4 C for two months, then germinated and grown to the four-leaf stage. These S/R F_1 plants were sprayed with a low use rate (0.4 kg a.e. ha^{-1}) formulated glyphosate (potassium salt, Roundup Weather Max, Monsanto Company, St. Louis, MO) to select for heterozygous resistant progeny, as apomixis may occur in Palmer amaranth (Trucco

et al. 2007). One resistant F_1 male was selected for hand crossing to one resistant F_1 female to generate a hand-pollinated F_2 through half-sibling mating. Both parents of the hand-pollinated F_2 were sampled for DNA extraction (see below). Pollination bags were placed over the female inflorescences prior to emergence and pollen from the resistant male was applied by hand daily for two weeks. A different resistant female from the S/R F_1 was placed next to a different resistant male from the S/R F_1 to generate an F_2 population through open-pollination of half-siblings. Seeds from the female plants were harvested by hand, labeled, and stored at 4 C for two months.

Sequencing

Primers were designed from waterhemp (*A. tuberculatus*) EPSPS gene sequence (P. Tranel, unpublished data). The primer sets EPSF1 x EPSR1, EPSF5 x EPSR5, and EPSF6 x EPSR6 (Table 2 - 1) were used to amplify overlapping regions of the EPSPS gene from resistant and susceptible cDNA (see below). The EPSF1 x EPSR1 PCR product contains the Pro106 codon. Seven resistant individuals and two susceptible individuals were used. The EPSF1 x EPSR1 PCR product was ligated into pGEM-T Easy plasmids (Promega, Madison, WI). Plasmids were transformed into *E. coli* cells, and transformed cells were cultured overnight in liquid LB media. Plasmids were isolated and sequenced using the M13F and M13R primers. The EPSF5 x EPSR5 and EPSF6 x EPSR6 PCR products were isolated for sequencing without cloning. The sequences were assembled into consensus contigs using Lasergene v. 7.0 SeqMan (DNASTAR, Inc., Madison, WI).

One resistant individual cDNA sample from the 8 HAT time-course treatment (see below) was amplified using the EPSF1 x EPSR1 primer set and the PCR product

was cloned as described previously. Eighteen clones were selected for sequencing with the M13F primer. Sequences were analyzed for single-nucleotide polymorphisms (SNPs) using SeqMan. The consensus sequence contig was aligned with the resistant and susceptible contigs using ClustalW2 (European Bioinformatics Institute).

DNA Blots

DNA blot analysis of resistant and susceptible individuals was conducted by extracting genomic DNA with a modified CTAB method (Murray and Thompson 1980). Three resistant and three susceptible genomic DNA samples were further purified using a $CsCl_2$ gradient. Approximately 10 µg total genomic DNA was digested with each restriction enzyme. Hybridization and detection were performed with either the Amersham ECL nucleic acid labeling and detection system (GE Life Sciences) or ^{32}P-dCTP (Perkin-Elmer, Waltham, MA) labeled probe (Random Prime DNA labeling kit, Sigma-Aldrich, St. Louis, MO). Blots were hybridized with probes for EPSPS and ALS synthesized from susceptible cDNA.

Time-course treatment

Seeds from the resistant and susceptible populations were germinated in small pots and grown to the four-leaf stage. Five plants each of resistant and susceptible were sampled for one 4-mm leaf disc for in-vivo measurement of background absorbance in the leaf disc shikimate assay of Shaner et al. (2005) and one leaf disc for RNA extraction (see below). Four plants of resistant and susceptible were then treated with 0.4 kg ae ha^{-1} glyphosate. Eight h after treatment (HAT) all plants were again sampled for one leaf disc for shikimate measurement and one leaf disc for RNA extraction. The 8 HAT leaf disc samples were taken from both sides of the midvein at the base of the leaf, and the 0 HAT

leaf disc samples were taken distal to the 8 HAT location. One plant each of resistant and susceptible was untreated. The experiment was conducted twice.

Genetic populations experiment

Seeds of the hand-pollinated and open-pollinated F_2 populations, along with resistant and susceptible seeds, were germinated and grown in small pots. Fifty-four plants of each F_2 and twelve plants of resistant and susceptible were grown to the four-leaf stage. One leaf of each plant was used for an in-vivo leaf disc shikimate accumulation assay (Shaner et al. 2005) with glyphosate concentrations of 0 and 250 μM in a 10 mM ammonium phosphate buffer. A shikimate standard curve was used to calculate the ng shikimate μL^{-1} accumulation above the background level. Each plant was assayed in duplicate. One leaf from each plant was sampled for genomic DNA extraction and one leaf for RNA extraction (see below).

Extraction of RNA and DNA

Tissue samples were immediately frozen in liquid nitrogen, ground in a 1.5 mL microcentrifuge tube, and stored at -80 C until use. 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. The DNA concentration was adjusted to 1 ng μL^{-1} in sterile HPLC grade water. RNA was extracted using Trizol reagent and procedures (Invitrogen, Carlsbad, CA), dissolved in sterile HPLC water, quantified using a NanoDrop spectrophotometer, checked for quality and integrity by gel electrophoresis, and checked for genomic DNA contamination by PCR.

cDNA Synthesis

Palmer amaranth RNA (200 ng for time course treatments and 700 ng from each of 20 F_2 individuals) was used for cDNA synthesis with oligo-DT primers and the Verso cDNA kit (Thermo Scientific, Wilmington, DE). This kit includes a DNAse treatment. Final cDNA volume was 20 µL.

Quantitative PCR

Quantitative real-time PCR was used to measure EPSPS genomic copy number relative to ALS and cDNA expression level of EPSPS relative to ALS. Primer efficiency curves were conducted for each primer set using a 1X, 1/5X, 1/25X, and 1/125X dilution series of resistant genomic DNA. The primer sets EPSF1 x EPSR8 (195 bp product) and ALSF2 x ALSR2 (118 bp product) (Table 2 - 1) were used to measure EPSPS genomic copy number relative to ALS. ALS was used as a low-copy control gene with known monogenic inheritance in other *Amaranthus* species (Trucco et al. 2005).

Triplicate genomic DNA templates (10 ng) were amplified in a 25 µL reaction volume using Syber-Green master mix (Bio-Rad Laboratories, Hercules, CA) by the following thermoprofile on a MyiQ real-time PCR detection system (Bio-Rad): 95 C for 15 minutes, then 30 cycles of 95 C for 30 sec and 60 C for 1 min. Real-time fluorescence data were captured during the amplification cycles. Melt-curve analysis was conducted by holding the samples at 95 C for 5 min, reducing the temperature to 55 C for 5 min, followed by increasing the temperature by 0.5 C every 10 sec to 95 C. Negative controls consisting of template with no primers and primers with no template were included. Threshold cycles were calculated using iCycler iQ v. 3.1 (Bio-Rad). Gene expression levels were measured using the same PCR protocol with 1 µL cDNA as template.

RESULTS AND DISCUSSION

Sequencing

Alignment of consensus sequences from seven resistant and two susceptible individuals showed one polymorphism found in all resistant sequences at position 1172 in the alignment contig (Figure 2 - 1). Susceptible plants have a guanine while resistant plants have an adenine, resulting in a change from arginine to lysine at position 316 of the plant EPSPS sequence. Analysis of SNPs in 18 clones from one resistant individual showed that six of the cDNA clones had a unique SNP (Table 2 - 2). Two of the SNPs resulted in a non-synonymous mutation. The polymorphic amino acid residues at positions 67 and 215 are found in published EPSPS sequences from plant species that are susceptible to glyphosate.

None of these polymorphisms have been shown previously to confer target-site glyphosate resistance. The glyphosate-susceptible rice EPSPS sequence has a lysine at position 316, suggesting that Lys316 does not confer glyphosate resistance. The amino acid changes at positions 67 and 215 are common in other plant EPSPS sequences, also suggesting that they do not confer resistance.

DNA Blots

Hybridization with partial-length EPSPS probes synthesized from susceptible cDNA showed that resistant genomic DNA samples had much stronger hybridization

intensity than susceptible samples (Figures 2 - 2A, 2B, and 2C). Hybridization with ALS probes showed that resistant and susceptible plants had similar ALS copy number (Figure 2 - 2D). The non-radioactive hybridization and labeling method did not provide sufficient sensitivity to detect the EPSPS band in susceptible samples (Figure 2 - 2A). Radioactive labeling was sensitive enough to detect EPSPS bands in susceptible samples (Figures 2 - 2B and 2C). All resistant samples had much higher hybridization intensity with EPSPS probes, but the total number of bands was similar for resistant and susceptible. Because the DNA blots indicated that resistant plants had a higher copy number, EPSPS expression level and genomic copy number were measured using quantitative PCR.

Quantitative PCR

Quantitative PCR is a convenient technique to estimate gene amplification, providing no unspecific PCR products are produced and primers are efficient across a wide range of template dilutions (Weill et al. 2000). Melt-curve analysis showed that no primer-dimers formed with either primer set. The melting peak for products of both primer sets was 86.0 C. Primer efficiency and slope for EPSPS was 100.2% and -3.318, and 103.8% and -3.235 for ALS. No amplification products were observed in any negative controls.

Time-course treatment

Resistant and susceptible plants were sampled for shikimate accumulation and RNA prior to and 8 HAT with 0 or 0.4 kg ha^{-1} glyphosate. Susceptible plants accumulated shikimate 8 HAT with glyphosate (Figure 2 - 3) while resistant plants did not. EPSPS expression in cDNA was measured relative to ALS using quantitative PCR.

Resistant plants had 35 times higher cDNA EPSPS expression relative to ALS than susceptible plants (Figure 2 - 3), and expression was unaffected by glyphosate treatment or time after glyphosate treatment. EPSPS expression was only normalized against one control gene, ALS, but results were very consistent between replications and experimental runs for an EPSPS:ALS relative expression ratio of approximately 1 in susceptible plants and a ratio of 30 to 40 in resistant plants. Resistant plants always had higher relative EPSPS expression, and relative expression levels were not affected 8 HAT in resistant or susceptible, despite the clear physiological effect of glyphosate application in susceptible plants.

Genetic populations experiment

All plants sampled from the resistant population had increased genomic EPSPS copy number compared to plants from the susceptible population (Figure 2 - 4). All susceptible plants had genomic EPSPS copy number relative to ALS within the range of 1.0 to 1.3, and resistant plants varied from a relative copy number of 5 to over 160. Due to the logarithmic nature of PCR amplification, there is inherent error in threshold cycle estimation. Due to the consistent 1:1 C_t ratio for EPSPS and ALS in susceptible plants, we have reason to be confident in the large relative copy number estimates based on Ct of EPSPS and ALS in resistant plants.

All susceptible plants accumulated shikimate at 250 μM glyphosate, while only the lowest relative copy number plant from the resistant population accumulated shikimate at the same dose. One resistant plant with a relative EPSPS copy number of 65 had some shikimate accumulation above background levels (Figure 2 – 4). The quantity of shikimate accumulation was substantially less than that observed in susceptible plant

samples. The in-vivo leaf disc assay is subject to sampling error with respect to the specific tissue sampled. Leaf disc samples were taken from the same positions on each leaf, but it is possible that variation in glyphosate sensitivity exists within a leaf.

ALS copy number was inherited and stable in both F_2 populations, while individuals varied greatly for EPSPS relative copy number (Figures 2 - 5A and 5B). It appears that for the majority of individuals in the F_2, the 250 μM in-vivo assay rate was not enough to overwhelm the resistance mechanism. Generally, all F_2 individuals with increased EPSPS relative copy number did not accumulate shikimate at 250 μM glyphosate, indicating they were resistant, although a few individuals with greater than 20 relative copies accumulated some shikimate at 250 μM glyphosate. Because gene over-expression can lead to gene silencing, the low levels of shikimate accumulation could be due to variation in silencing within a leaf sample resulting in small variations in resistance levels. Some individuals had less shikimate at 250 μM than the background (0 μM) sample, so some of the increase relative to background could be due to lower than expected shikimate levels in the background sample. All F_2 individuals with one relative EPSPS copy were susceptible and clearly distinguishable by quantity of shikimate accumulation.

Expression and copy number correlation

Selected individuals from the F_2 and parental populations were measured for EPSPS expression relative to ALS. There was a strong correlation ($r = 0.76$) between EPSPS relative copy number and EPSPS relative expression level (Figure 2 - 6). Plants with an EPSPS copy number relative to ALS of 1 and similar EPSPS relative expression accumulated shikimate at 250 μM, while individuals with increased EPSPS relative copy

number and relative expression did not (Figure 2 - 7). There was a strong correlation between EPSPS relative copy number and glyphosate resistance (Figure 2 – 5).

The inset histograms in Figures 2 - 5A and 5B show an approximately continuous distribution for EPSPS relative copy number. Given the relative copy numbers measured for the F_2 hand-pollinated population parents (18 in the male parent and 39 in the female parent), the relative copy number distribution is not consistent with a monogenic inheritance model of gene amplification blocks. Unequal sister chromatid exchange during meiosis resulting in gain and loss of copies within the repeat could explain the continuous distribution of EPSPS relative copy number.

Amplification of genes encoding enzymes targeted by pesticides has been shown to confer pesticide resistance in other species. Copy number of metabolic genes conferring resistance to organophosphate insecticides in *Culex* mosquitoes was measured using quantitative PCR (Callaghan et al. 1998; Paton et al. 2000). Resistant mosquitoes had approximately 80 times more copies (Paton et al. 2000). Amplification levels of organophosphate metabolic resistance genes varied between individuals over time (Callaghan et al. 1998), correlating with variation in organophosphate selection pressure. Two mosquito strains that had a unique sequence polymorphism, indicating they descended from the same strain, varied by 13-fold in esterase gene amplification. Amplified esterase genes in *Myzus* aphid populations had the same flanking sequence around the tandem array (Field and Devonshire 1997). The arrangement of the repeats was consistent with unequal sister chromatid exchange, a model that predicts expansion of gene amplifications due to unequal exchanges. Both examples indicate that loss or gain of gene copies can occur rapidly, possibly due to unequal sister-chromatid exchange

during meiosis (Callaghan et al. 1998; Field and Devonshire 1997). Unequal crossing over was proposed as a mechanism for tandem amplification of *Rps1* resistance genes in soybean (Bhattacharyya et al. 2005). Another example of amplified resistance genes is *Rp1* in maize, where rust resistance gene paralogs range from 1 to >50 in different maize lines (Smith et al. 2004). Tandemly arrayed genes are more likely to occur in regions of higher recombination and along chromosome arms in Arabidopsis (Zhang and Gaut 2003).

Alternatively, the inset histograms in Figures 2 - 2A and 2B could show that most individuals have either 1, 20, 40, or 60 relative EPSPS copies, the approximate possible combinations of alleles from the parents (Figure 2 - 8), and that the continuous distribution is due to error in C_t estimates. The histograms could show an over-abundance of 40 relative EPSPS copy plants if seeds were produced through apomixis resulting in clones of the maternal (heterozygous) genotype (Trucco et al. 2007). Fewer numbers of 1 and 60 relative EPSPS copy plants were observed (Figure 2 - 5A) than would be expected under a monogenic inheritance model where each possible genotype in the F_2 (Figure 2 - 8) should have a frequency of 0.25.

Over-expression of EPSPS mRNA due to gene amplification is heritable and correlates with resistance to 250 µM glyphosate in an in-vivo leaf disc assay. Additional EPSPS copies may confer higher levels of resistance on individuals. Resistance was only measured at one glyphosate concentration in this experiment, so the additive effect of additional EPSPS copies on resistance is presumed. The advantage of additional EPSPS genomic copies was shown to be increased EPSPS expression (Figure 2 - 6). The mRNA over-expression should result in over-production of EPSPS, and higher over-expression

should result in higher overall resistance levels. Over-expression as a molecular glyphosate resistance mechanism has been demonstrated in cell culture selection, but never before in a weed population. The continuous application of low glyphosate rates with no rotation to other control methods at the original field site (Culpepper et al. 2006) may have selected for individuals with a previously existing EPSPS gene amplification, or the EPSPS gene amplification may have occurred during selection. As evidence for the latter, plants from the field population are highly variable for EPSPS relative copy number (Figure 2 - 4).

The gene amplification could be selected as an additive trait, where additional copies confer additional resistance, and increasing rates selects for individuals with increased copy number. Recurrent selection in waterhemp (*A. tuberculatus*) resulted in increased glyphosate tolerance (Zelaya and Owen 2005), which could be indicative of a similar gene amplification phenomenon taking place in a few generations. One individual in the Palmer amaranth F_2 had a higher relative EPSPS copy number than the sum of the relative copy number from both parents (Figure 2 - 5A), indicating that additional copies can be gained in one generation. Some individuals in the F_2 also have lower relative EPSPS copy number than the lower relative copy number male parent, indicating that some copies can be lost in one generation. Both results are consistent with unequal chromatid pairing at meiosis and subsequent gain or loss of duplicated copies. Unequal chromatid pairing may be the mechanism producing gene amplification in glyphosate resistant carrot cell lines (Murata et al. 1998).

Both parental amplification blocks (of approximately 20 and 40) provide an increase in EPSPS expression (Figure 2 - 6) and having them together gives an additive

effect to increase expression further. It is unknown at this time whether a mutation such as Pro106 is present within the duplicated locus. It is reasonable to expect that the native over-expression in Palmer amaranth would not have the tissue specificity of the FMV 35S promoter (Chen et al. 2006) and that over-expression is adequate in reproductive tissues, especially given the reproductive fitness of treated resistant Palmer amaranth plants.

Agronomic practices which involve additional control measures in addition to or in replacement of glyphosate would reduce the selection advantage conferred by the gene amplification and the gene amplification could decrease in frequency within the resistant populations over time. EPSPS gene amplification and increased EPSPS activity may have a fitness penalty, as the amplification is usually lost in the absence of glyphosate selection pressure in cell culture (Pline-Srnic 2006). This suggests that removing glyphosate selection pressure from Palmer amaranth populations could cause the EPSPS gene amplification to decrease in frequency. Additional questions remain, such as whether this resistance mechanism is common among other resistant Palmer amaranth populations or in other glyphosate resistant species. It should be determined whether the EPSPS gene amplification has occurred rapidly since the introduction of glyphosate-resistant cropping systems, or whether the gene amplification was present at a low level in the population prior to glyphosate application.

Gene amplification is an independent outcome of adaptive genetic change from point mutations and gene amplification occurs under stress, at least in *E. coli* (Hastings et al. 2004). Amplification is very common in all organisms, occurs under conditions where enhanced expression of the amplified genes is advantageous, can occur in response in

stress, and allows evolution of function (Hastings 2007). There are mechanisms by which amplification can be a stress response, but the organism has to be able to live through the stress without the amplification initially. Amplification is adaptive because it is a rapid, reversible response (at least in microbes). Young cell cultures did not always have EPSPS gene amplification, while the same lines did at older stages (Papanikou et al. 2004). It was suggested that early selection results in increased transcription, transcript stability, or enzyme stability, and with time, the controls that repair DNA breaks are lost and gene amplification can occur. It was also suggested that this process was unlikely to occur in whole plants under field selection (Papanikou et al. 2004). The observation that gene amplification occurs easily in plant tissue culture and not in normal cells could indicate that the basic genetic control over gene amplification is lost in cell culture. Palmer amaranth offers an opportunity to study loss of gene amplification control in whole plants. Relevant questions include the frequency of gene amplification across the genome and whether Palmer amaranth has rapidly evolved resistance to so many herbicides (Heap 2008) due to gene amplification.

REFERENCES

Baerson, S. R., D. J. Rodriguez, M. Tran, Y. M. Feng, N. A. Biest, and G. M. Dill. 2002a. Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Phys. 129:1265-1275.

Baerson, S. R., D. J. Rodriguez, N. A. Biest, M. Tran, J. S. You, R. W. Kreuger, G. M. Dill, J. E. Pratley, and K. J. Gruys. 2002b. Investigating the mechanism of glyphosate resistance in rigid ryegrass (*Lolium ridigum*). Weed Sci. 50:721-730.

Behrens, M. R., N. Mutlu, S. Chakraborty, R. Dumitru, W. Z. Jiang, B. J. LaVallee, P. L. Herman, T. E. Clemente, and D. P. Weeks. 2007. Dicamba resistance: Enlarging and preserving biotechnology-based weed management strategies. Science 316:1185-1188.

Bhattacharyya, M. K., N. N. Narayanan, H. Gao, D. K. Santra, S. S. Salimath, T. Kasuga, Y. Liu, B. Espinosa, L. Ellison, L. Marek, R. Shoemaker, M. Gijzen, and R. I. Buzzell. 2005. Identification of a large cluster of coiled coil-nucleotide binding site-leucine rich repeat-type genes from the *Rps1* region containing *Phytophthora* resistance genes in soybean. Theor. Appl. Gen. 111:75-86.

Boerboom, C. M., D. L. Wyse, and D. A. Somers. 1990. Mechanism of glyphosate tolerance in birdsfoot-trefoil (*Lotus corniculatus*). Weed Sci. 38:463-467.

Bradshaw, L. D., S. R. Padgette, S. L. Kimball, and B. H. Wells. 1997. Perspectives on glyphosate resistance. Weed Technol. 11:189-198.

Callaghan, A., T. Guillemaud, N. Makate, and M. Raymond. 1998. Polymorphisms and fluctuations in copy number of amplified esterase genes in *Culex pipiens* mosquitoes. Insect Mol. Biol. 7:295-300.

Chen, Y. C. S., C. Hubmeier, M. Tran, A. Martens, R. E. Cerny, R. D. Sammons, and C. CaJacob. 2006. Expression of CP4 EPSPS in microspores and tapetum cells of cotton (*Gossypium hirsutum*) is critical for male reproductive development in response to late-stage glyphosate applications. Plant Biotechnol. J. 4:477-487.

Culpepper, A. S., T. L. Grey, W. K. Vencill, J. M. Kichler, T. M. Webster, S. M. Brown, A. C. York, J. W. Davis, and W. W. Hanna. 2006. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci. 54:620-626.

Dill, G. M. 2005. Glyphosate-resistant crops: history, status and future. Pest Mgmt. Sci. 61:219-224.

Feng, P. C. C., M. Tran, T. Chiu, R. D. Sammons, G. R. Heck, and C. A. CaJacob. 2004. Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. Weed Sci. 52:498-505.

Field, L. M. and A. L. Devonshire. 1997. Structure and organization of amplicons containing the E4 esterase genes responsible for insecticide resistance in the aphid *Myzus persicae* (Sulzer). Biochem. J. 322:867-871.

Forlani, G., E. Nielsen, and M. L. Racchi. 1992. A glyphosate-resistant 5-enol-pyruvyl-shikimate-3-phosphate synthase confers tolerance to a maize cell line. Plant Sci. 85:9-15.

Harms, C. T., S. L. Armour, J. J. Dimaio, L. A. Middlesteadt, D. Murray, D. V. Negrotto, H. Thompsontaylor, K. Weymann, A. L. Montoya, R. D. Shillito, and G. C. Jen.

1992. Herbicide resistance due to amplification of a mutant acetohydroxy acid synthase gene. Mol. Gen. Genet. 233:427-435.

Hastings, P. J. 2007. Adaptive amplification. Crit. Rev. Biochem. Mol. Biol. 42:271-283.

Hastings, P. J., A. Slack, J. F. Petrosino, and S. M. Rosenberg. 2004. Adaptive amplification and point mutation are independent mechanisms: Evidence for various stress-inducible mutation mechanisms. Plos Biology 2:2220-2233.

Heap, I. 2008. The International Survey of Herbicide Resistant Weeds. Online. Available at www.weedscience.com (accessed October 21, 2008).

Herrmann, K. M. and L. M. Weaver. 1999. The shikimate pathway. Ann. Rev. Plant Phys. Plant Mol. Biol. 50:473-503.

Jones, J. D., P. B. Goldsbrough, and S. C. Weller. 1996. Stability and expression of amplified EPSPS genes in glyphosate resistant tobacco cells and plantlets. Plant Cell Rep. 15:431-436.

Kaundun, S. S., I. A. Zelaya, R. P. Dale, A. J. Lycett, P. Carter, K. R. Sharpies, and E. McIndoe. 2008. 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.

Murata, M., J. H. Ryu, S. Caretto, D. Rao, H. S. Song, and J. M. Widholm. 1998. Stability and culture medium limitations of gene amplification in glyphosate resistant carrot cell lines. J. Plant Phys. 152:112-117.

Murray, M. G. and W. F. Thompson. 1980. Rapid isolation of high molecular-weight plant DNA. Nuc. Acids Res. 8:4321-4325.

Ng, C. H., R. Wickneswary, S. Salmijah, Y. T. Teng, and B. S. Ismail. 2004. Glyphosate resistance in *Eleusine indica* (L.) Gaertn. from different origins and polymerase chain reaction amplification of specific alleles. Aust. J. Ag. Res. 55:407-414.

Norsworthy, J. K., G. M. Griffith, R. C. Scott, K. L. Smith, and L. R. Oliver. 2008. Confirmation and control of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in Arkansas. Weed Technol. 22:108-113.

Padgette, S. R., D. B. Re, G. F. Barry, D. E. Eichholtz, X. Delannay, R. L. Fuchs, G. M. Kishore, and R. T. Fraley. 1996. New weed control opportunities: Development of soybeans with a Roundup Ready gene, *In* S. O. Duke, ed. Herbicide-resistant crops: Agricultural, environmental, economic, regulatory, and technical aspects. CRC Press, Inc., Boca Raton, FL.

Papanikou, E., J. E. Brotherton, and J. M. Widholm. 2004. Length of time in tissue culture can affect the selected glyphosate resistance mechanism. Planta 218:589-598.

Paton, M. G., S. Karunaratne, E. Giakoumaki, N. Roberts, and J. Hemingway. 2000. Quantitative analysis of gene amplification in insecticide-resistant *Culex* mosquitoes. Biochem. J. 346:17-24.

Pline-Srnic, W. 2006. Physiological mechanisms of glyphosate resistance. Weed Technol. 20:290-300.

Powles, S. B. 2008. Evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Mgmt. Sci. 64:360-365.

Powles, S. B. and C. Preston. 2006. Evolved glyphosate resistance in plants: Biochemical and genetic basis of resistance. Weed Technol. 20:282-289.

Sammons, R. D., D. C. Heering, N. Dinicola, H. Glick, and G. A. Elmore. 2007. Sustainability and stewardship of glyphosate and glyphosate-resistant crops. Weed Technol. 21:347-354.

Schmid, J. and N. Amrhein. 1995. Molecular organization of the shikimate pathway in higher plants. Phytochem. 39:737-749.

Schonbrunn, E., S. Eschenburg, W. A. Shuttleworth, J. V. Schloss, N. Amrhein, J. N. S. Evans, and W. Kabsch. 2001. Interaction of the herbicide glyphosate with its target enzyme 5-enolpyvuvylshikimate 3-phosphate synthase in atomic detail. Proc. Nat. Acad. Sci. 98:1376-1380.

Shah, D. M., R. B. Horsch, H. J. Klee, G. M. Kishore, J. A. Winter, N. E. Tumer, C. M. Hironaka, P. R. Sanders, C. S. Gasser, S. Aykent, N. R. Siegel, S. G. Rogers, and R. T. Fraley. 1986. Engineering herbicide tolerance in transgenic plants. Science 233:478-481.

Shaner, D. L., T. Nadler-Hassar, W. B. Henry, and C. H. Koger. 2005. A rapid in vivo shikimate accumulation assay with excised leaf discs. Weed Sci. 53:769-774.

Sidhu, R. S., B. G. Hammond, R. L. Fuchs, J. N. Mutz, L. R. Holden, B. George, and T. Olson. 2000. Glyphosate-tolerant corn: The composition and feeding value of grain from glyphosate-tolerant corn is equivalent to that of conventional corn (*Zea mays* L.). J. Agric. Food Chem. 48:2305-2312.

Smith, S. M., A. J. Pryor, and S. H. Hulbert. 2004. Allelic and haplotypic diversity at the *Rp1* rust resistance locus of maize. Genetics 167:1939-1947.

Steckel, L. E., C. L. Main, A. T. Ellis, and T. C. Mueller. 2008. Palmer amaranth (*Amaranthus palmeri*) in Tennessee has low level glyphosate resistance. Weed Technol. 22:119-123.

Steinrucken, H. C. and N. Amrhein. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic-acid 3-phosphate synthase. Biochem. Biophys. Res. Comm. 94:1207-1212.

Suh, H., A. G. Hepburn, A. L. Kriz, and J. M. Widholm. 1993. Structure of the amplified 5-enolpyruvylshikimate-3-phosphate synthase gene in glyphosate-resistant carrot cells. Plant Mol. Biol. 22:195-205.

Trucco, F., M. R. Jeschke, A. L. Rayburn, and P. J. Tranel. 2005. Promiscuity in weedy amaranths: High frequency of female tall waterhemp (*Amaranthus tuberculatus*) x smooth pigweed (*A. hybridus*) hybridization under field conditions. Weed Sci. 53:46-54.

Trucco, F., D. Zheng, A. J. Woodyard, J. R. Walter, T. C. Tatum, A. L. Rayburn, and P. J. Tranel. 2007. Nonhybrid progeny from crosses of dioecious amaranths: Implications for gene-flow research. Weed Sci. 55:119-122.

Vencill, W. K., T. L. Grey, A. S. Culpepper, T. A. Gaines, and P. Westra. 2008. Herbicide-resistance in the Amaranthaceae. J. Plant Dis. Protect. Special Issue XXI:41-44.

Weill, M., C. Berticat, N. Raymond, and C. Chevillon. 2000. Quantitative polymerase chain reaction to estimate the number of amplified esterase genes in insecticide-resistant mosquitoes. Anal. Biochem. 285:267-270.

Widholm, J. M., A. R. Chinnala, J. H. Ryu, H. S. Song, T. Eggett, and J. E. Brotherton. 2001. Glyphosate selection of gene amplification in suspension cultures of 3 plant species. Physiol. Plant. 112:540-545.

Zelaya, I. A. and M. D. K. Owen. 2005. Differential response of *Amaranthus tuberculatus* (Moq ex DC) JD Sauer to glyphosate. Pest Mgmt. Sci. 61:936-950.

Zhang, L. Q. and B. S. Gaut. 2003. Does recombination shape the distribution and evolution of tandemly arrayed genes (TAGs) in the *Arabidopsis thaliana* genome? Genome Res. 13:2533-2540.

TABLES

Table 2 - 1. Primers used to amplify 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS) genes from Palmer amaranth cDNA and genomic DNA.

Primer Name	5' to 3' sequence
EPSF1	ATGTTGGACGCTCTCAGAACTCTTGGT
EPSR1	GTCATAAGTTTCAATGGCGGTGG
EPSF5	GCCAAGAACACAAAGCGAAATTCAGAG
EPSR5	TCTTTACCAACAGGAAACAGACCACCAC
EPSF6	CAGGGAATCATCTGGAAGGAAACATTTG
EPSR6	CTATTAGTCTCAAATCAAAACCTTCGGCG
EPSR7	GCAAGAGTCATAGCAACATCTGGC
EPSR8	TGAATTTCCTCCAGCAACGGCAA
EPSF9	GCTCTCTGGATCGGTTAGTA
ALSF2	GCTGCTGAAGGCTACGCT
ALSR2	GCGGGACTGAGTCAAGAAGTG
ALSR1	TCCACCCTAATCGTAGCCAACTC

Table 2 - 2. Sequence polymorphisms found in different 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) alleles amplified from one individual glyphosate-resistant Palmer amaranth cDNA using primers EPSF1 and EPSR1, along with amino acid position for non-synonymous mutations in the plant EPSPS sequence. Contig position refers to the Rclones contig in Figure 2 - 1, multiple sequence alignment.

Contig Position	Reference Nucleotide	Clone Number	Sequence Change	Amino Acid Change	Plant EPSPS Position
110	A	15	GAT to GGT	Asp to Gly	67
171	A	9	AAA to AAG	Lys (silent)	
312	G	20	GGG to GGA	Gly (silent)	
351	A	4	GTA to GGT	Val (silent)	
354	T	4	GAT to GAC	Asp (silent)	
399	A	13	AAA to AAG	Lys (silent)	
408	T	4	CTT to CTG	Leu (silent)	
438	G	4	TCG to TCA	Ser (silent)	
549	A	6	ACA to ACG	Thr (silent)	
554	A	4	AAG to AGG	Lys to Arg	215

FIGURES

Figure 2 - 1. Alignment of full-length consensus 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) sequences from resistant (R) and susceptible (S) Palmer amaranth individuals and partial-length consensus sequence from 18 cDNA clones isolated from one resistant individual 8 h after treatment with 0.4 kg ha^{-1} glyphosate. Asterisks indicate nucleotides in all sequences are the same. Start and stop codons are indicated with a box.

```
Rconsensus      GAATATGGCTCAGCTACTACCATCAACAATGGTGTCCATACTGGTCAATTGCACCATACT 60
Sconsensus      GAATATGGCTCAGCTACTACCATCAACAATGGTGTCCATACTGGTCAATTGCACCATACT 60
Rclones         ------------------------------------------------------------

Rconsensus      TTACCCAAAACCCAGTTACCCAAATCTTCAAAAACTCTTAATTTTGGATCAAACTTGAGA 120
Sconsensus      TTACCCAAAACCCAGTTACCCAAATCTTCAAAAAYTCTTAATTTTGGATCAAACTTGAGA 120
Rclones         ------------------------------------------------------------

Rconsensus      ATTTCTCCAAAGTTCATGTCTTTAACCAATAAAAGAGTTGGTGGGCAATCATCAATTGTT 180
Sconsensus      ATTTCTCCAAAGTTCATGTCTTTAACCAATAAAAGAGTTGGTGGGCAATCATCAATTGTT 180
Rclones         ------------------------------------------------------------

Rconsensus      CCCAAGATTCAAGCTTCTGTTGCTGCTGCAGCTGAGAAACCTTCATCTGTCCCAGAAATT 240
Sconsensus      CCCAAGATTCAAGCTTCTGTTGCTGCTGCAGCTGAGAAACCTTCATCTGTCCCAGAAATT 240
Rclones         ------------------------------------------------------------

Rconsensus      GTGTTACAACCCATCAAAGAGATCTCTGGTACTGTTCAATTGCCTGGGTCAAAGTCTTTA 300
Sconsensus      GTGTTACAACCCATCAAAGAGATCTCTGGTACTGTTCAATTGCCTGGGTCAAAGTCTTTA 300
Rclones         ------------------------------------------------------------

Rconsensus      TCCAATCGAATCCTTCTTTTAGCTGCTTTG-TCTGAGGGCACAACAGTGGTCGACAACTT 359
Sconsensus      TCCAATCGAATCCTTCTTTTAGCTGCTTTG-TCTGAGGGCACAACAGTGGTCGACAACTT 359
Rclones         ------------CCTTTAKGGGCGAATTGGGCCCGACGTCGCA----TGCTCC-CGGCCG 43
                            * * *    **   ** *  * ** * * **    ** **  *  *

Rconsensus      GCTGTATAGTGATGATATTCTTTATATGTTGGACGCTCTCAGAACTCTTGGTTTAAAAGT 419
Sconsensus      GCTGTATAGTGATGATATTCTTTATATGTTGGACGCTCTCAGAACTCTTGGTTTAAAAGT 419
Rclones         CCATGGCGGCCGCGGGAATTCGATTATGTTGGACGCTCTCAGAACTCTTGGTTTAAAAGT 103
                 *      *    *  * *     ************************************

Rconsensus      GGAGGATGATAGTACAGCCAAAAGGGCAGTCGTAGAGGGTTGTGGTGGTCTGTTTCCTGT 479
Sconsensus      GGAGGATGATAGTACAGCCAAAAGGGCAGTCGTAGAGGGTTGTGGTGGTCTGTTTCCTGT 479
Rclones         GGAGGATGATAGTACAGCCAAAAGGGCAGTCGTAGAGGGTTGTGGTGGTCTGTTTCCTGT 163
                ************************************************************

Rconsensus      TGGTAAAGATGGAAAGGAAGAGATTCAACTTTTCCTTGGTAATGCAGGAACAGCGATGCG 539
Sconsensus      TGGTAAAGATGGAAAGGAAGAGATTCAACTTTTCCTTGGTAATGCAGGAACAGCGATGCG 539
Rclones         TGGTAAAGATGGAAAGGAAGAGATTCAACTTTTCCTTGGTAATGCAGGAACAGCGATGCG 223
                ************************************************************

Rconsensus      CCCATTGACAGCTGCGGTTGCCGTTGCTGGAGGAAATTCAAGTTATGTGCTTGATGGAGT 599
Sconsensus      CCCATTGACAGCTGCGGTTGCCGTTGCTGGAGGAAATTCAAGTTATGTGCTTGATGGAGT 599
Rclones         CCCATTGACAGCTGCGGTTGCCGTTGCTGGAGGAAATTCAAGTTATGTGCTTGATGGAGT 283
                ************************************************************
```

```
Rconsensus      ACCAAGAATGAGGGAGCGCCCCATTGGGGATCTGGTAGCAGGTCTAAAGCAACTTGGTTC 659
Sconsensus      ACCAAGAATGAGGGAGCGCCCCATTGGGGATCTGGTAGCAGGTCTAAAGCAACTTGGTTC 659
Rclones         ACCAAGAATGAGGGAGCGCCCCATTGGGGATCTGGTAGCAGGTCTAAAGCAACTTGGTTC 343
                ************************************************************

Rconsensus      AGATGTAGATTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAAGGAGG 719
Sconsensus      AGATSTAGATTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAAGGAGG 719
Rclones         AGATGTAGATTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAAGGAGG 403
                **** ********************************************************

Rconsensus      CCTTCCAGGGGGCAAGGTCAAGCTCTCTGGATCGGTTAGTAGCCAATATTTAACTGCACT 779
Sconsensus      CCTTCCAGGGGGCAAGGTCAAGCTCTCTGGATCGGTTAGTAGCCAATATTTAACTGCACT 779
Rclones         CCTTCCAGGGGGCAAGGTCAAGCTCTCTGGATCGGTTAGTAGCCAATATTTAACTGCACT 463
                ************************************************************

Rconsensus      TCTCATGGCTACTCCTTTGGGTCTTGGAGACGTGGAGATTGAGATAGTTGATAAATTGAT 839
Sconsensus      TCTCATGGCTACTCCTTTGGGTCTTGGAGACGTGGAGATTGAGATAGTTGATAAATTGAT 839
Rclones         TCTCATGGCTACTCCTTTGGGTCTTGGAGACGTGGAGATTGAGATAGTTGATAAATTGAT 523
                ************************************************************

Rconsensus      TTCTGTACCGTATGTTGAAATGACAATAAAGTTGATGGAACGCTTTGGAGTATCCGTAGA 899
Sconsensus      TTCTGTACCGTATGTTGAAATGACAATAAAGTTGATGGAACGCTTTGGAGTATCCGTAGA 899
Rclones         TTCTGTACCGTATGTTGAAATGACAATAAAGTTGATGGAACGCTTTGGAGTATCCGTAGA 583
                ************************************************************

Rconsensus      ACATAGTGATAGTTGGGACAGGTTCTACATTCGAGGTGGTCAGAAATACAAATCTCCTGG 959
Sconsensus      ACATAGTGATAGTTGGGACAGGTTCTACATTCGAGGTGGTCAGAAATACAAATCTCCTGG 959
Rclones         ACATAGTGATAGTTGGGACAGGTTCTACATTCGAGGTGGTCAGAAATACAAATCTCCTGG 643
                ************************************************************

Rconsensus      AAAGGCATATGTTGAGGGTGATGCTTCAAGTGCTAGCTACTTCCTAGCCGGAGCCGCCGT 1019
Sconsensus      AAAGGCATATGTTGAGGGTGATGCTTCAAGTGCTAGCTACTTCCTAGCCGGAGCCGCCGT 1019
Rclones         AAAGGCATATGTTGAGGGTGATGCTTCAAGTGCTAGCTACTTCCTAGCCGGAGCCGCCGT 703
                ************************************************************

Rconsensus      CACTGGTGGGACTGTCACTGTCAAGGGTTGTGGAACAAGCAGTTTACAGGGTGATGTAAA 1079
Sconsensus      CACTGGTGGGACTGTCACTGTCAAGGGTTGTGGAACAAGCAGTTTACAGGGTGATGTAAA 1079
Rclones         CACTGGTGGGACTGTCACTGTCAAGGGTTGTGGAACAAGCAGTTTACAGGGTGATGTAAA 763
                ************************************************************

Rconsensus      ATTTGCCGAAGTTCTTGAGAAGATGGGTTGCAAGGTCACCTGGACAGAGAATAGTGTAAC 1139
Sconsensus      ATTTGCCGAAGTTCTTGAGAAGATGGGTTGCAAGGTCACCTGGACAGAGAATAGTGTAAC 1139
Rclones         ATTTGCCGAAGTTCTTGAGAAGATGGGTTGCAAGGTCACCTGGACAGAGAATAGTGTAAC 823
                ************************************************************

Rconsensus      TGTTACTGGACCACCCAGGGATTCATCTGGAAAGAAACATCTGCGTGCTATCGACGTCAA 1199
Sconsensus      TGTTACTGGACCACCCAGGGATTCATCTGGAAGGAAACATCTGCGTGCTATCGACGTCAA 1199
Rclones         TGTTACTGGACCACCCAGGGATTCATCTGGAAAGAAACATCTGCGTGCT---------- 872
                ******************************** ****************

Rconsensus      CATGAACAAAATGCCAGATGTTGCTATGACTCTTGCAGTTGTTGCCTTGTATGCAGATGG 1259
Sconsensus      CATGAACAAAATGCCAGATGTTGCTATGACTCTTGCAGTTGTTGCCTTGTATGCAGATGG 1259
Rclones         ------------------------------------------------------------

Rconsensus      GCCCACCGCCATCAGAGATGTGGCTAGCTGGAGAGTGAAGGAAACCGAACGGATGATTGC 1319
Sconsensus      GCCCACCGCCATCAGAGATGTGGCTAGCTGGAGAGTGAAGGAAACCGAACGGATGATTGC 1319
Rclones         ------------------------------------------------------------

Rconsensus      CATTTGCACAGAACTGAGAAAGCTTGGGGCAACAGTTGAGGAAGGATCTGATTACTGTGT 1379
Sconsensus      CATYTGCACAGAACTGAGAAAGCTTGGGGCAACAGTTGAGGAAGGATCTGATTACTGTGT 1379
Rclones         ------------------------------------------------------------

Rconsensus      GATCACTCCGCCTGAAAAGCTAAACCCCACCGCCATTGAAACTTATGACGATCACCGAAT 1439
Sconsensus      GATCACTCCGCCYGAAAAGCTAAACCCCACCGCCATTGAAACTTATGACGATCACCGAAT 1439
Rclones         ------------------------------------------------------------
```

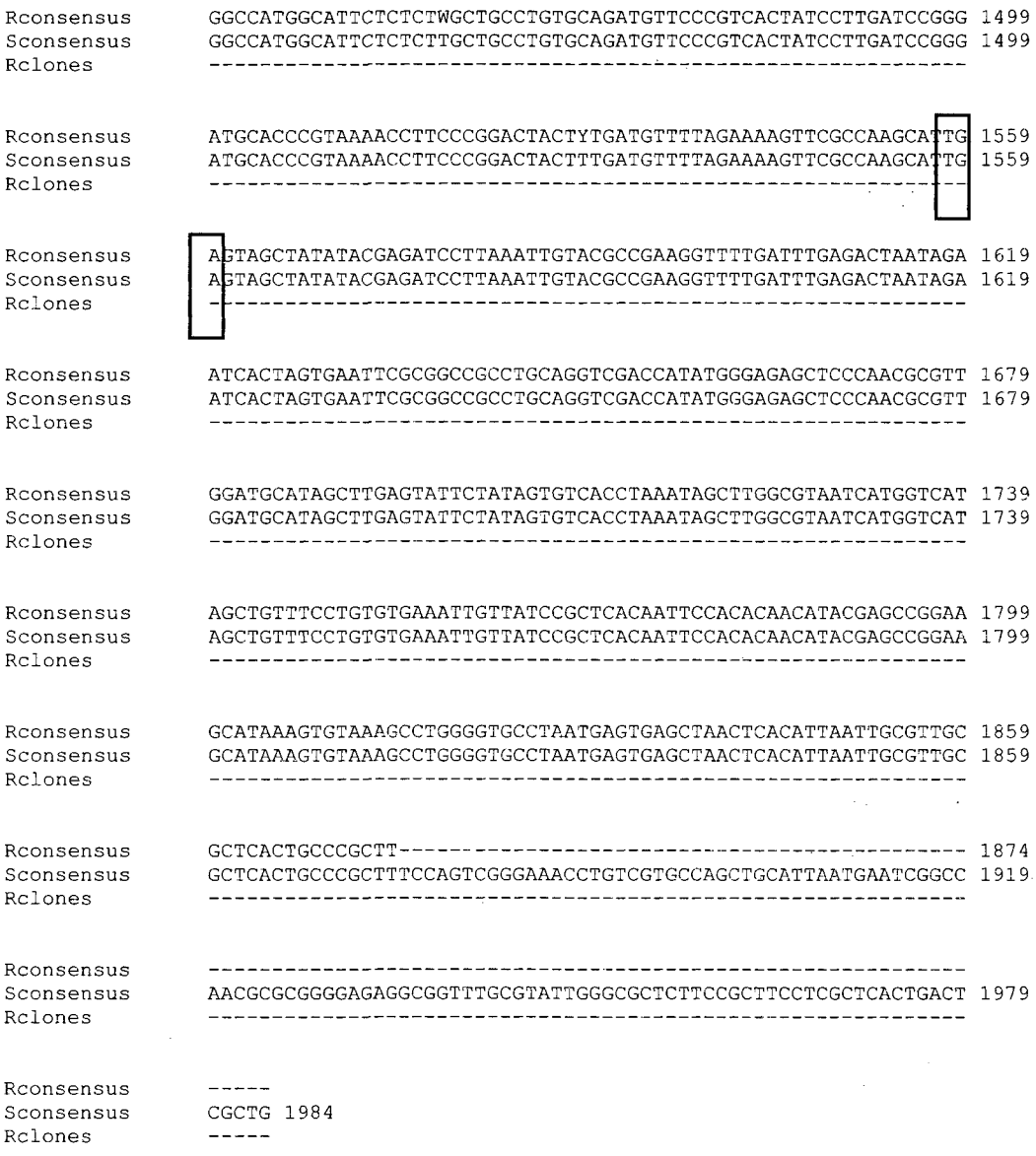
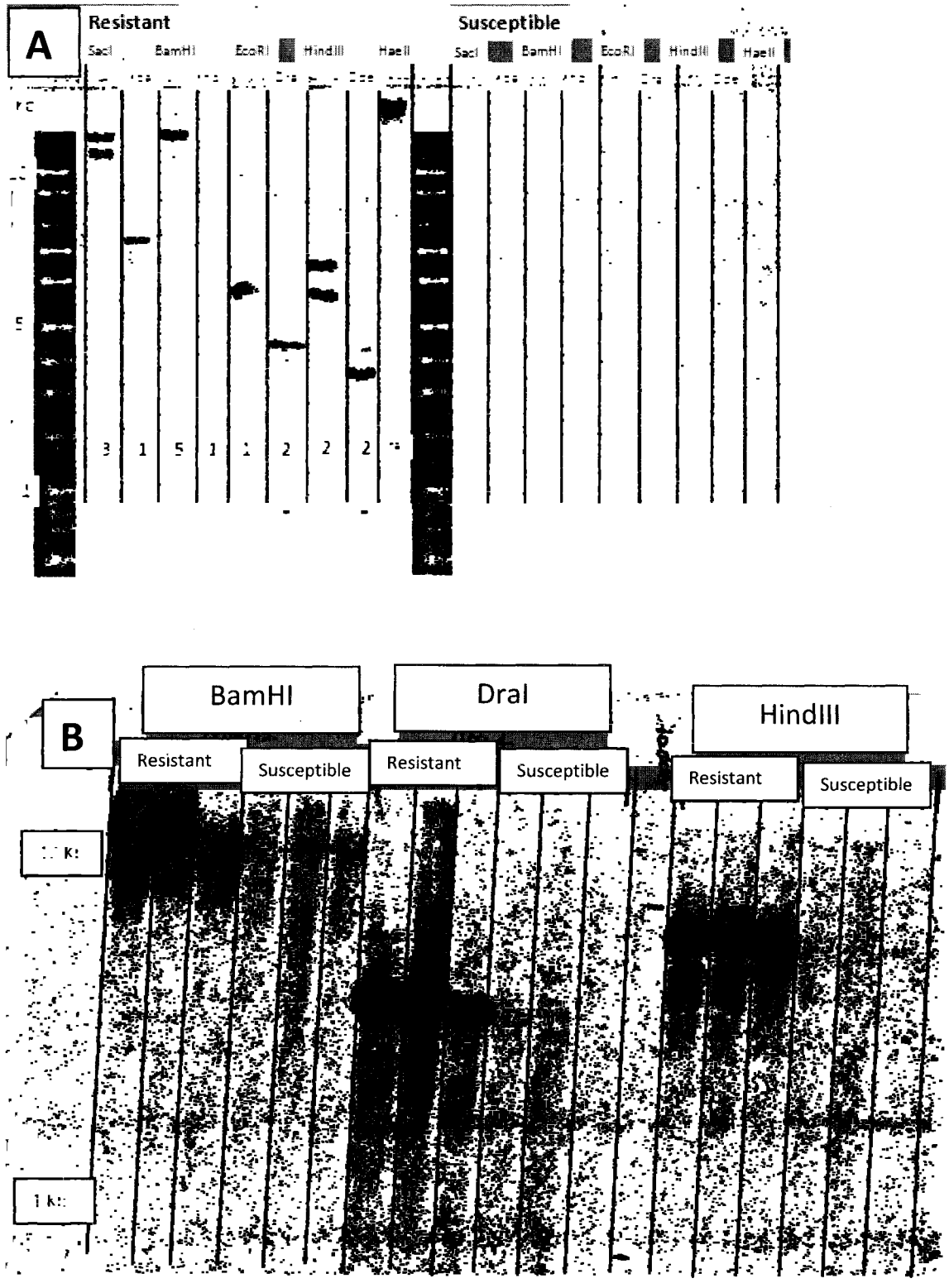

Rconsensus GGCCATGGCATTCTCTCTWGCTGCCTGTGCAGATGTTCCCGTCACTATCCTTGATCCGGG 1499
Sconsensus GGCCATGGCATTCTCTCTTGCTGCCTGTGCAGATGTTCCCGTCACTATCCTTGATCCGGG 1499
Rclones ----------
Rconsensus ATGCACCCGTAAAACCTTCCCGGACTACTYTGATGTTTTAGAAAAGTTCGCCAAGCATTG 1559
Sconsensus ATGCACCCGTAAAACCTTCCCGGACTACTTTGATGTTTTAGAAAAGTTCGCCAAGCATTG 1559
Rclones ----------
Rconsensus AGTAGCTATATACGAGATCCTTAAATTGTACGCCGAAGGTTTTGATTTGAGACTAATAGA 1619
Sconsensus AGTAGCTATATACGAGATCCTTAAATTGTACGCCGAAGGTTTTGATTTGAGACTAATAGA 1619
Rclones ----------
Rconsensus ATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTT 1679
Sconsensus ATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTT 1679
Rclones ----------
Rconsensus GGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCAT 1739
Sconsensus GGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCAT 1739
Rclones ----------
Rconsensus AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA 1799
Sconsensus AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA 1799
Rclones ----------
Rconsensus GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGC 1859
Sconsensus GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGC 1859
Rclones ----------
Rconsensus GCTCACTGCCCGCTT---------- 1874
Sconsensus GCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCC 1919
Rclones ----------
Rconsensus ----------
Sconsensus AACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACT 1979
Rclones ----------
Rconsensus -----
Sconsensus CGCTG 1984
Rclones -----

Figure 2 - 2. DNA blots of resistant and susceptible Palmer amaranth genomic DNA. A) One genomic template of resistant and susceptible extracted using the CTAB method, digested with SacI, XbaI, BamHI, XhoI, EcoRI, DraI, HindIII, DdeI, and HaeII, and hybridized with non-radioactive labeled probe for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) synthesized from susceptible cDNA. B) Three genomic DNA samples each of resistant and susceptible, extracted with the CTAB method and purified using a $CsCl_2$ gradient, digested with BamHI, DraI, and HindIII, and hybridized with ^{32}P-dCTP labeled probe for EPSPS synthesized with primers EPSF9 x EPSR7 from susceptible cDNA. C) The same DNA blot as B, except the blot was washed with a higher stringency wash. D) Three genomic DNA samples each of resistant and susceptible, extracted with the CTAB method and purified using a $CsCl_2$ gradient, digested with BamHI, DraI, and HindIII, and hybridized with ^{32}P-dCTP labeled probe for acetolacate synthase (ALS) synthesized with primer ALSF2 x ALSR1 from susceptible cDNA.

(continued next page)

Figure 2 – 2 continued.



continued next page

Figure 2 – 2 continued

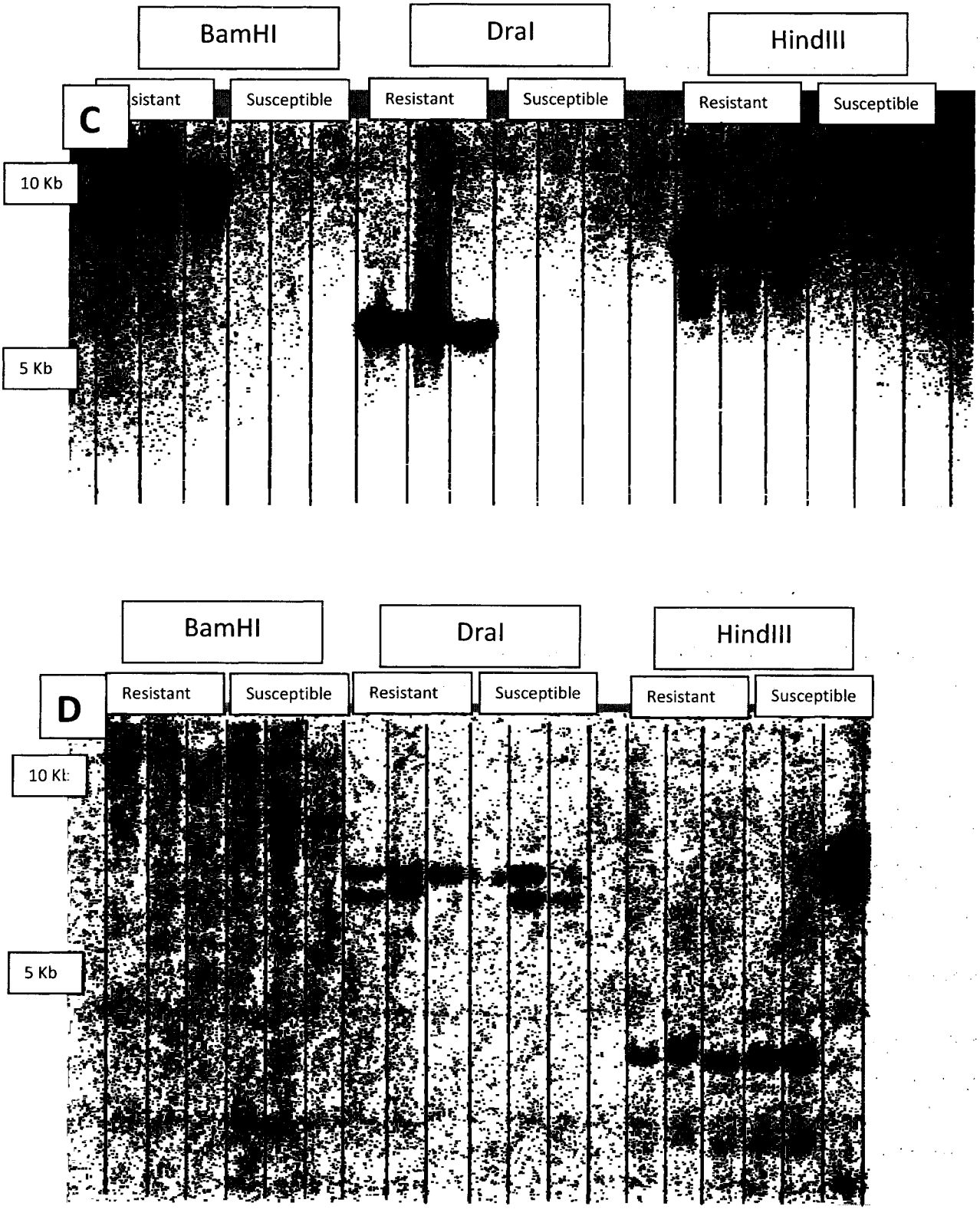


Figure 2 - 3. 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) expression relative to acetolactate synthase (ALS) measured using quantitiative PCR on cDNA and shikimate accumulation 8 hours after treatment (HAT) with 0.4 kg ha^{-1} glyphosate in resistant and susceptible Palmer amaranth. Data points are means and standard errors of two runs.

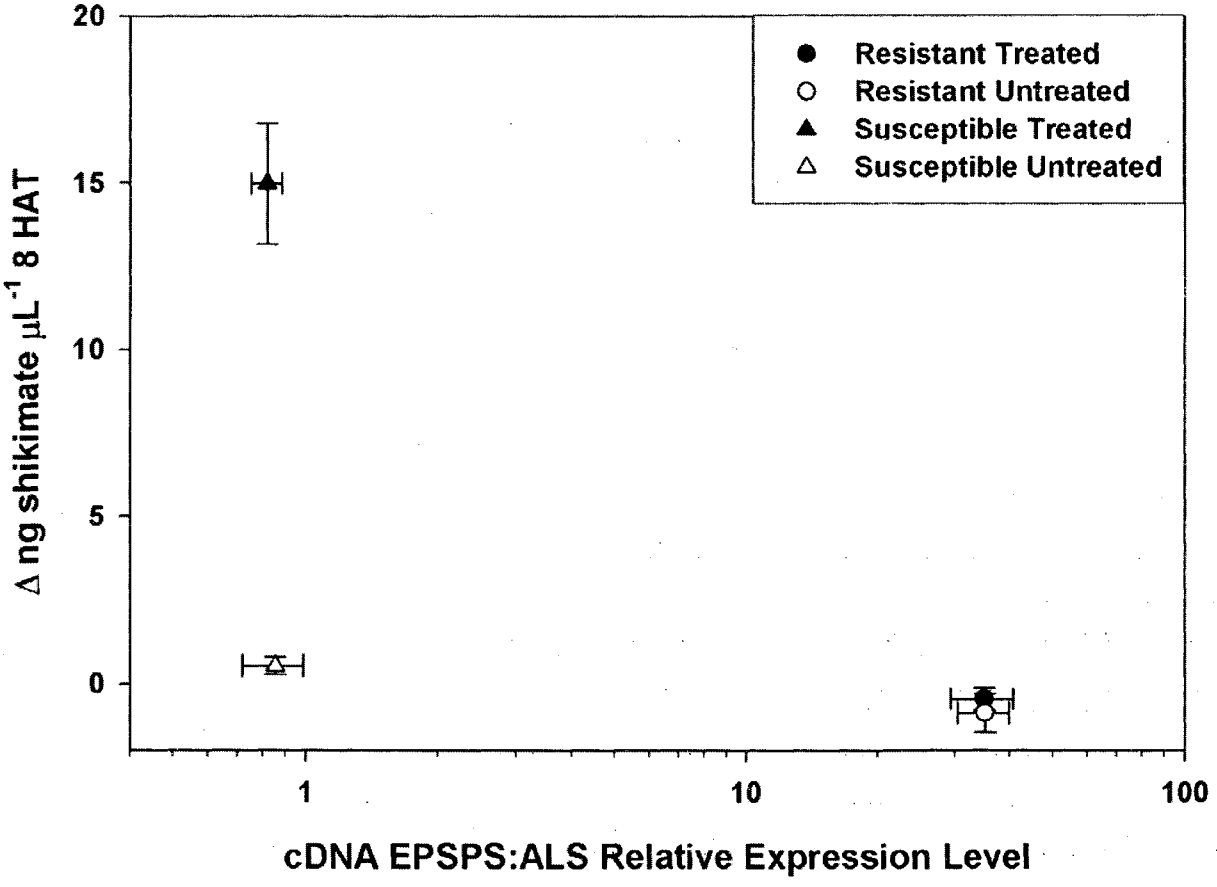


Figure 2 - 4. Genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative to acetolactate synthase (ALS) measured using quantitative PCR on genomic DNA and accumulation of shikimate above background levels following incubation in 250 μM glyphosate in an in vivo leaf disc assay in resistant and susceptible Palmer amaranth plants.

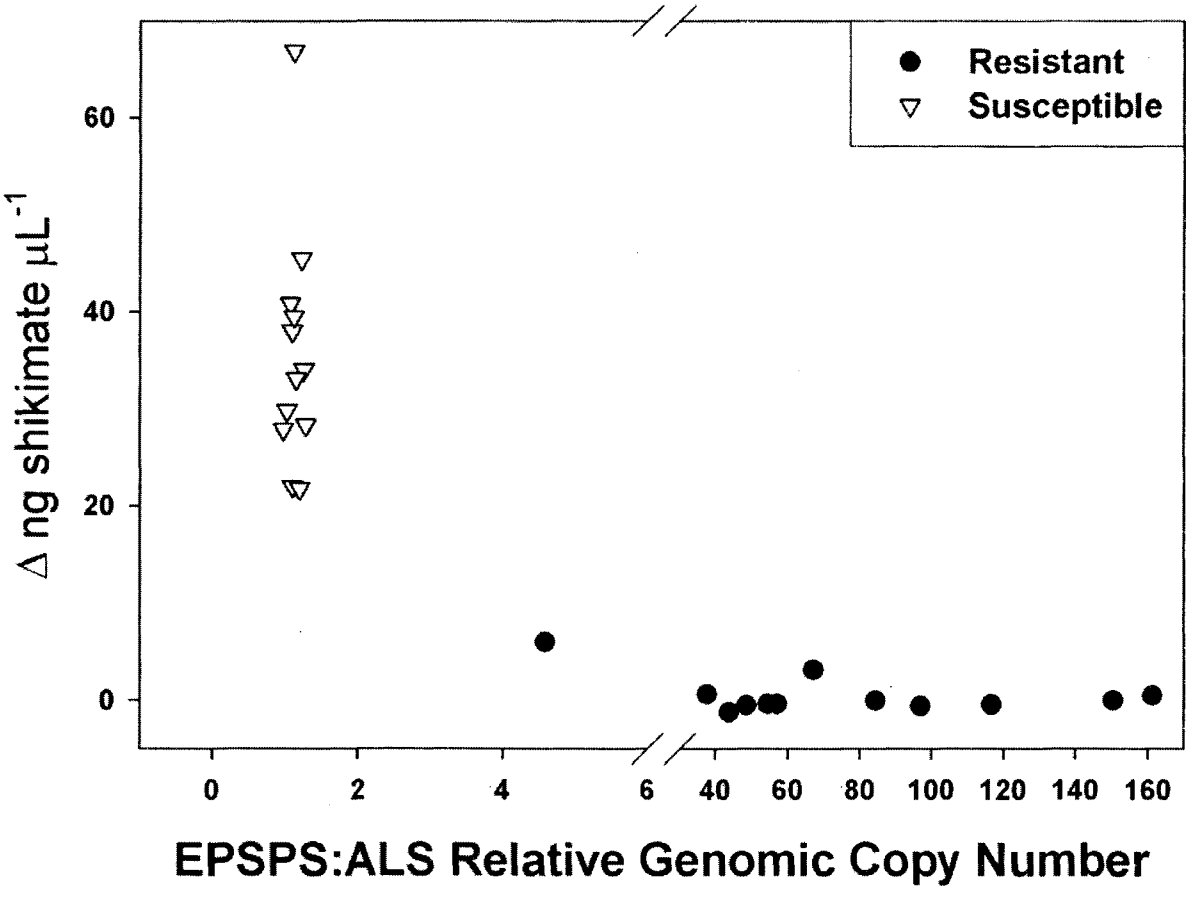


Figure 2 - 5. Genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative to acetolactate synthase (ALS) measured using quantitative PCR on genomic DNA, accumulation of shikimate above background levels following incubation in 250 μM glyphosate in an in vivo leaf disc assay, with insets containing relative copy number histograms in Palmer amaranth F_2 populations generated using A) hand pollination (F_1 male parent 18 relative EPSPS copies and female parent 39 relative EPSPS copies) and B) open pollination (parental relative copy number not measured).

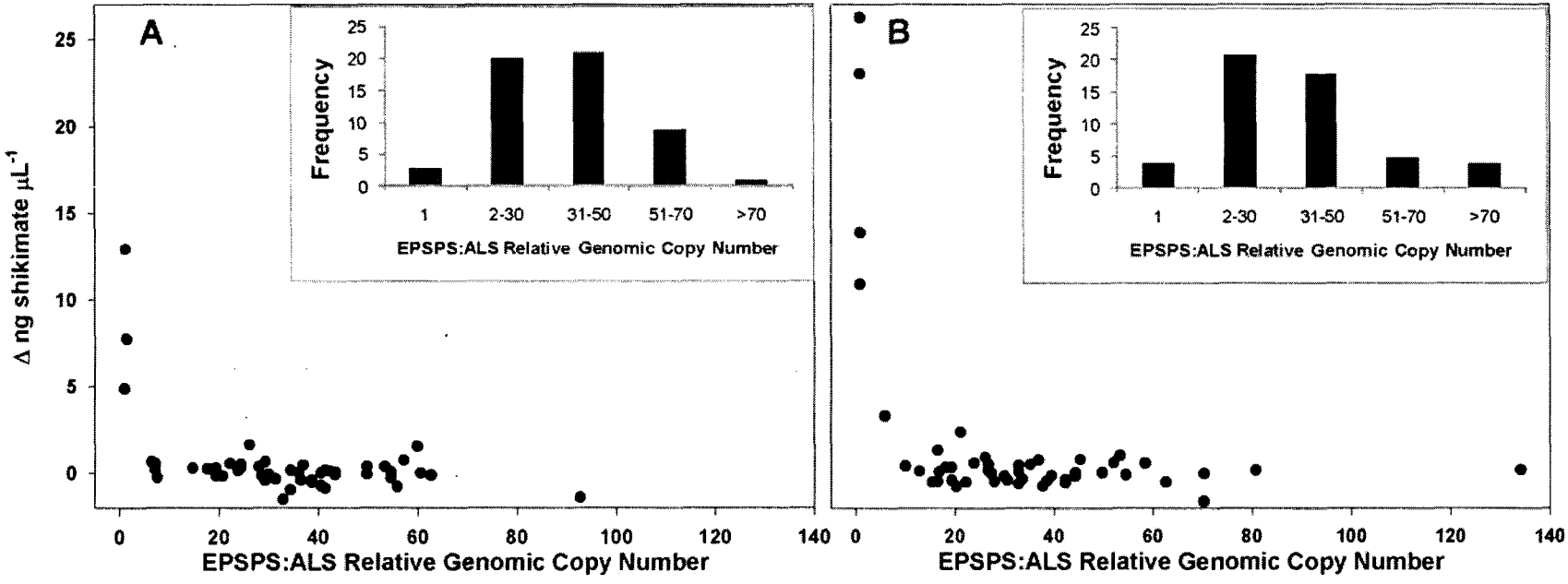


Figure 2 - 6. Correlation between 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) genomic copy number relative to acetolactate synthase (ALS) genomic copy number as determined by quantitative PCR, and EPSPS cDNA expression level relative to ALS as determined by quantitative PCR in selected F_2, confirmed resistant and confirmed susceptible Palmer amaranth plants.

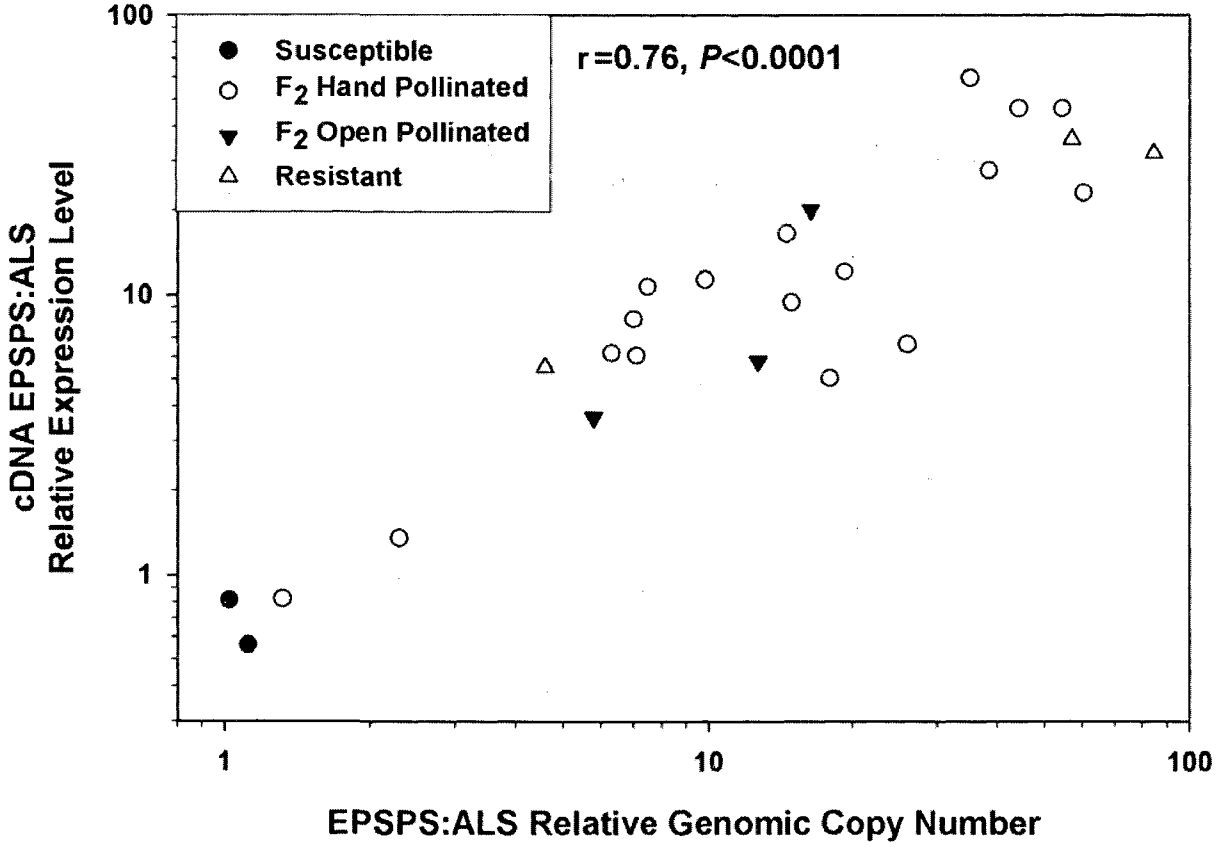


Figure 2 - 7. 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) genomic copy number relative to acetolactate synthase (ALS) copy number, EPSPS cDNA expression level relative to ALS, and shikimate accumulation above background at 250 μM glyphosate in selected F_2, resistant and susceptible Palmer amaranth plants.

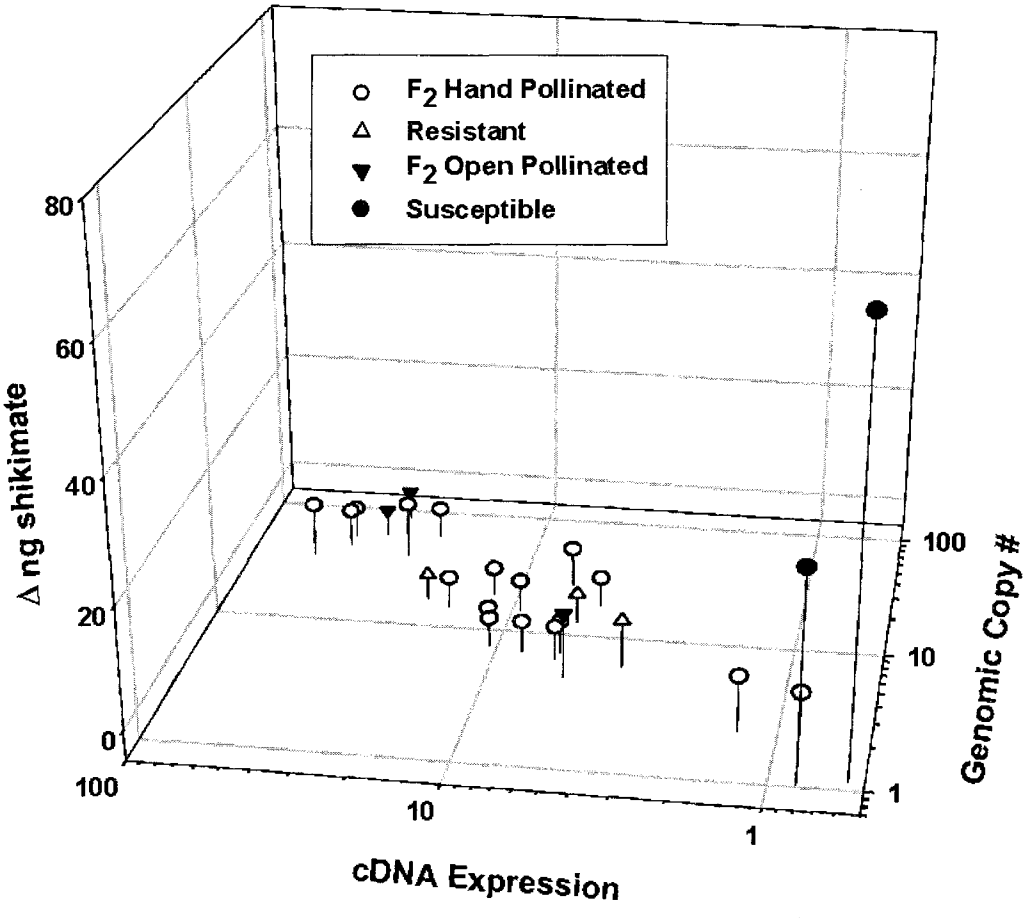
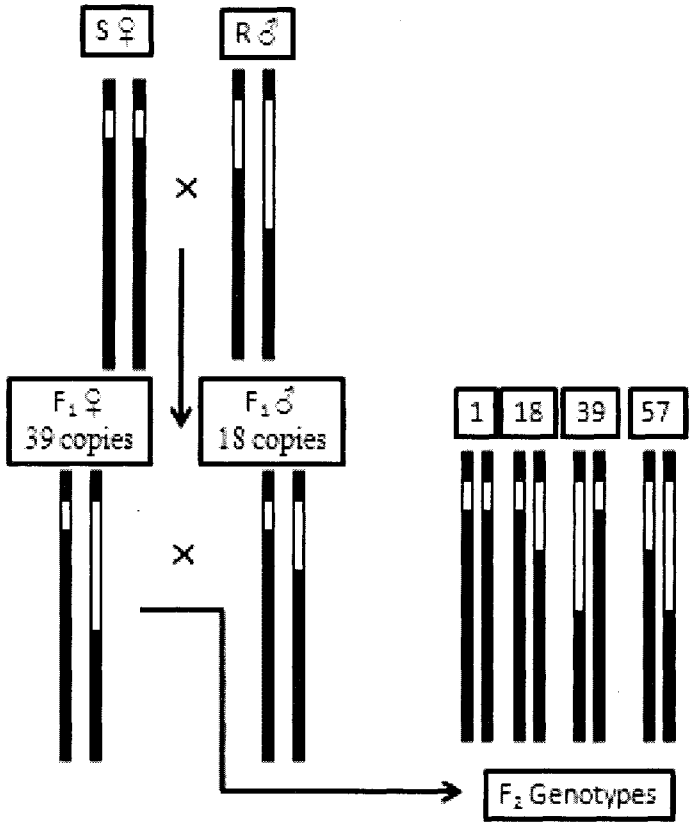


Figure 2 - 8. Proposed model of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene amplification in Palmer amaranth and probable F_2 genotypes, based on F_1 parent EPSPS:acetolactate synthase relative genomic copy number. The resistant male parent is assumed to have one block of 18 relative EPSPS copies and one block of 39 relative copies. Black lines represent chromosomes and white block represent the size of the gene amplification locus.



CHAPTER 3: GENE TRANSFER TO RELATED *AMARANTHUS* SPECIES

Potential for transfer of glyphosate resistance from *Amaranthus palmeri* to other *Amaranthus* species.

Todd A. Gaines, Philip Westra, Christopher Preston, Sarah Ward, Dale L. Shaner, Bekir Bukun, Jan E. Leach, and Scott J. Nissen.

ABSTRACT

The evolution of glyphosate resistant *Amaranthus palmeri* from Georgia is a major agronomic problem. Because evolved glyphosate resistance is relatively rare, one concern is that the resistance trait could be transferred to related *Amaranthus* species. Transfer of herbicide resistance traits has been documented between other *Amaranthus* species and between *A. palmeri* and *A. tuberculatus*. The objective of this study was to determine the risk of hybridization and glyphosate resistance gene transfer from *A. palmeri* to *A. hybridus*, *A. retroflexus*, *A. powellii*, *A. spinosus*, and *A. tuberculatus*. A field study with resistant male *A. palmeri* and recipient plants of the other species at 1 and 3 m distance was conducted in 2006 and 2007. Hand crosses were also made in the greenhouse with *A. palmeri* pollen to each of the other five species. Glyphosate application (0.4 kg ha^{-1}) was used to screen for resistant progeny from the crosses. Surviving plants were sampled for DNA to conduct quantitative PCR for 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene amplification and for species-diagnostic restriction polymorphisms in internal transcribed spacer (ITS) and acetolactate synthase (ALS) genes. Hybridization with *A. spinosus* occurred in both years of the field study and in hand crosses, with average frequency ranging from <0.01% to 1.4%. Hybrids with *A. spinosus* were either monoecious or dioecious, monoecious plants produced seed through self-pollination, and the F_2 progeny segregated for resistance. Hybridization occurred in the 2007 field study with *A. hybridus* (<0.01%) and *A. tuberculatus* (0.08% and 0.19% for two accessions), all hybrid plants were dioecious, and none produced seed. Hybridization occurred in *A. spinosus* plants at 3 m from the pollen source. The highest risk for glyphosate resistance gene transfer from *A. palmeri* is to *A. spinosus*.

Herbicide resistance in the genus *Amaranthus* is a major agronomic problem (Vencill et al. 2008), including recent reports of glyphosate resistant *A. palmeri* in the southeast United States (Culpepper et al. 2006; Heap 2008; Norsworthy et al. 2008; Steckel et al. 2008). The number of glyphosate resistant weed species has increased recently, but glyphosate resistance is still less frequent than resistance to herbicides in other mode of action categories (Heap 2008). One relevant concern is that the resistance trait could transfer to related *Amaranthus* species (Culpepper et al. 2006). Inter-specific hybridization is known to occur in the *Amaranthus* genus, making classification of *Amaranthus* species difficult (Sauer 1950).

Chromosome number could be one predictive variable for hybridization between *Amaranthus* species. *A. spinosus* has chromosome number of 2n=34 and was shown to hybridize with *A. dubius* (Grant 1959a), and with *A. hybridus* (Greizerstein and Poggio 1995), but hybrids from both crosses were mostly sterile. The original report of chromosome number in *A. palmeri* is 2n=34 (Grant 1959b), and for *A. tuberculatus* is 2n=32. A discrepancy exists for *A. palmeri* chromosome number. Rayburn et al. (2005) reported 2n=32 for *A. palmeri* and *A. tuberculatus*, and also found that *A. palmeri* and *A. spinosus* have similar sized genomes (0.95 and 1.1 pg per 2C nucleus, respectively), both on the smaller end of the species examined (e.g., A. tuberculatus was 1.42 pg per 2C nucleus). Grant (1959c) reported the following chromosome numbers: *A. hybridus* (2n=32), *A. palmeri* (2n=34), *A. powellii* (2n=34), *A. retroflexus* (2n=34), *A. spinosus*

(2n=34), and *A. tuberculatus* (2n=32). Based on reported inter-specific hybridizations, equal chromosome number is not a prerequisite for hybridization, but it seems logical that equal chromosome number could make subsequent genetic introgression easier. Greizerstein and Poggio (1995) hypothesized that the genus *Amaranthus* contains a basic set of 8 and a basic set of 9 chromosomes. Therefore, inter-specific hybridization between species with 2n=34 would have (8+8) and (9+9) and could reasonably be expected to have more fertile hybrids with other species with 2n=34 than those with 2n=32 (8+8) and (8+8).

Three monoecious species (*A. retroflexus*, *A. powellii*, and *A. hybridus*) used in this study are chiefly self-pollinated (Murray 1940), but all the species used in this study are capable of out-crossing through wind pollination. Murray (1940) concluded that males are the heterogametic sex in dioecious *Amaranthus* species, and that dioecy was dominant to monoecy in inter-specific crosses. *A. spinosus* is monoecious with imperfect flowers (Grant 1959a). Both *A. tuberculatus* and *A. palmeri* are dioecious (Grant 1959b). Successful crosses have been made between species used in this study, including *A. hybridus* x *A. powellii*, *A. hybridus* x *A. retroflexus*, *A. retroflexus* x *A. powellii*, *A. retroflexus* x *A. spinosus*, *A. hybridus* x *A. spinosus*, *A. retroflexus* x *A. tuberculatus*, and *A. tuberculatus* x *A. hybridus* (Murray 1940). The *A. spinosus* flowering habit was lost in crosses with *A. retroflexus* (maintained *A. retroflexus* type), and *A. spinosus* crosses with *A. hybridus* produced no pattern of male-female flower arrangement, but did result in imperfect flowers. However, with the exception of *A. tuberculatus* [e.g., (Franssen et al. 2001; Steinau et al. 2003; Trucco et al. 2007; Wetzel et al. 1999b)], *A. palmeri* has not

been examined for inter-specific compatibility with other important weedy *Amaranthus* species.

Since these other five species (*A. hybridus*, *A. powellii*, *A. retroflexus*, *A.spinosus*, and *A. tuberculatus*) have been shown to cross in various combinations, a reasonable hypothesis is that *A. palmeri* will also cross with each of the five species. Genomic constitution of *A. palmeri* and *A. tuberculatus* hybrids may be unstable, as indicated by presence of AFLP bands in hybrids not found in either parent (Steinau et al. 2003; Wetzel et al. 1999b). The only previous reports of confirmed hybridization within *Amaranthus* under field experimental conditions are between *A. hybridus* and *A. tuberculatus* (Trucco et al. 2005a; Trucco et al. 2005b). A genetic diversity study using molecular markers found that *A. palmeri* and *A. spinosus* clustered together, and predicted that inter-specific hybridization between these two species may be possible (Wassom and Tranel 2005). The objective of this study was to determine the risk of glyphosate resistance gene transfer from *A. palmeri* to five other *Amaranthus* species.

MATERIALS AND METHODS

Field Pollen-Mediated Gene Transfer Study

Glyphosate resistance gene transfer frequency from *A. palmeri* to five other *Amaranthus* species was measured under field conditions. Seeds of *A. hybridus*, *A. palmeri*, *A. powellii* ssp. *powellii*, *A. powellii* ssp. *bouchonii*, *A. retroflexus*, *A. tuberculatus*, and *A. spinosus* were obtained from the United States Department of

Agriculture – Agriculture Research Service National Plant Germplasm System North Central Regional Plant Introduction Station in Ames, IA (listed in Table 3 - 1). Seeds of *A. hybridus* and *A. tuberculatus* were obtained from Kansas State University, and seeds of glyphosate susceptible and resistant *A. palmeri* were obtained from the University of Georgia. Plants were propagated by germinating seeds on blotter paper, transplanting to small pots, and then re-potting in 4 L pots with commercial potting soil and slow-release granular fertilizer (Osmocote, Scotts Company). Plants were grown in a greenhouse under natural light conditions supplemented with 400 W sodium halide lamps to provide a 14-h daylength. Daytime temperatures were approximately 24 C and nighttime temperatures were approximately 18 C.

Plants were moved to a field site spatially isolated from agronomic crop production prior to flowering initiation to establish a plot consisting of one row of 20 male resistant *A. palmeri* plants in the middle, with a parallel row 1 m on either side consisting of two plants of each species, and a parallel row 3 m on either side. Plants were spaced 0.5 m apart. Plants were maintained by watering daily. The field experiment was conducted in 2006 and 2007. Male *A. palmeri* flowered first and continued flowering during the entire flowering duration of the other species. Female *A. palmeri* were included in the plot design to measure intra-specific pollination. The plot area was maintained by mowing and hand-weeding to eliminate weed competition. Height measurements were recorded for each plant.

Plants were harvested at physiological maturity in the fall. Seeds were manually threshed from the plants. Total above-ground dry biomass and seed biomass were

recorded. Seeds were stored at 4 C until use. A 0.1 g aliquot of seeds from each plant was counted to estimate total seed number.

Screening for Hybrids from Field Study

Each seed sample was screened in two runs with five replications for glyphosate-resistant hybrids. One replication consisted of 1 g of seed. Seeds were spread on moistened potting soil in germination boxes and covered with a thin layer of potting soil. Sealed boxes were placed in a 4 C cold room for 7 d. The boxes were transferred to germination chambers for two cycles of the following temperate regime: 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 light. Germinated seedlings were spread onto moistened potting soil in 25 by 50 cm flats, placed in a greenhouse, and watered daily. Liquid fertilizer (20-20-20) was applied once.

The number of emerged plants was estimated by counting plants in two 1.5 cm wide strips along the length of each flat. Percent emergence was calculated by dividing the total number of emerged plants by the estimated seed number planted. When the seedlings had two true leaves, they were treated with 0.4 kg ae ha^{-1} commercially formulated glyphosate in a pressurized spray chamber calibrated to deliver 187 L ha^{-1} at 206 kPa. Plants were evaluated for survival 14 d after treatment. Surviving plants were considered as those that had grown new leaves and increased in height, and these surviving plants were considered putative hybrids.

Putative hybrids were transplanted and grown to maturity for evaluation of floral and vegetative morphology. Leaves from these plants were sampled for DNA extraction (see below). Putative hybrids were self-pollinated to determine whether seed would be

produced. Seed from self-pollinated plants was germinated and treated with 0.4 kg ae ha^{-1} glyphosate to check for resistance segregation in F_2 progeny (Hidayat et al. 2006).

Hand Crosses

Reciprocal hand crosses were made between *A. palmeri* and five *Amaranthus* species. All *A. palmeri* plants were confirmed as glyphosate resistant using the *in-vivo* leaf disc method of Shaner et al. (2005). Hand crosses using *A. palmeri* as a female parent were made using three female plants and one male of each species. Female inflorescences were bagged upon emergence and pollen was applied by hand daily for two wk. Hand crosses using *A. palmeri* as a male parent were made using four plants of each species and four male *A. palmeri* plants. Because pistillate flowers emerge and are receptive to pollen several days prior to the opening of staminate flowers, crosses can be made by heavily pollinating when the stigmas emerge (Murray 1940). Pollen from glyphosate resistant *A. palmeri* plants was applied to plants of the other species from the first stigma emergence and daily thereafter for two weeks.

Hand Crosses Evaluation

Seeds from female *A. palmeri* plants crossed with other species were germinated and grown to maturity. Emergence rates and vegetative and floral morphology were recorded. Seeds (5 g) from the five species pollinated by male *A. palmeri* were germinated and treated with 0.4 kg ae ha^{-1} glyphosate at the 2-3 leaf stage. Seed density was estimated by counting seeds in a 1 g aliquot of each seed sample and used to estimate percent emergence. Surviving plants were transplanted. Leaf tissue was sampled from each putative hybrid plant for DNA extraction (see below). Seed from self-pollinated

plants was germinated and treated with 0.4 kg ae ha^{-1} glyphosate to check for segregation of resistance in F_2 progeny (Hidayat et al. 2006).

Verification of Putative Hybrids

Genomic DNA was extracted from putative hybrid plants using a modified CTAB method (Murray and Thompson 1980). Amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene was used as a molecular marker to verify that putative hybrids had inherited the resistance trait. Quantitative PCR was used to measure EPSPS copy number relative to acetolactate synthase (ALS) copy number with the methods described in Chapter 2. This eliminated false positives, namely any susceptible plants of the other species that survived glyphosate treatment without being resistant. To ensure that putative hybrids were not *A. palmeri* seed contaminants, the ITS region was amplified using the method of Wetzel et al. (1999a) to confirm the presence of ITS alleles from both parental species for crosses with *A. hybridus*, *A. powellii*, *A. retroflexus*, and *A. spinosus*. Aliquots of the amplified ITS product were digested with BsaAI, HaeII, and/or XhoI, depending on the species. The ALS gene was amplified from putative hybrids with *A. tuberculatus* and digested with EcoRV to confirm the presence of ALS alleles from *A tuberculatus* (contains an EcoRV restriction site) and *A. palmeri* (lacks restriction site) (Tranel et al. 2002; Trucco et al. 2007). Data from initial screening results and from putative hybrid confirmation are presented.

Data Analysis

Phenotypic data were analyzed using ANOVA and comparisons were made using Fisher's LSD (SAS 2004). Means and standard errors were calculated for percent

emergence, initial screening percent survival, confirmed hybrids, and percent survival of putative hybrid progeny.

RESULTS AND DISCUSSION

Field Pollen-Mediated Gene Transfer Study

Plant height and total seed production were different in 2006 and 2007 (Table 3 - 1). Male *A. palmeri* plants shed pollen during the entire growing season both years, enabling overlap in pollination times with all other species.

Over 400,000 progeny from *Amaranthus* species in the field study were screened for glyphosate resistance. Resistant plants were found in progeny of susceptible female *A. palmeri* (Table 3 - 2), indicating that pollination was sufficient for intra-specific gene transfer up to 3 m distance. Emergence rates ranged from 20% to 96%, with the exception of *A. tuberculatus*, which had very low emergence (Table 3 - 2). Because *A. tuberculatus* is dioecious and no *A. tuberculatus* males were present, most of the seeds may have been non-viable. At least some progeny from all tested *Amaranthus* species survived glyphosate treatment, with the exception of *A. powellii* ssp. *bouchonii* and one *A. tuberculatus* accession (Table 3 - 2). All surviving putative hybrids were examined using multiple techniques, with the exception of *A. spinosus* hybrids from 2007. Due to the large number of putative hybrids, a sub-sample of putative *A. spinosus* hybrids was examined.

Some putative hybrids were rejected because it was determined that they were instead *A. palmeri* plants that had contaminated a seed sample, based on ITS or ALS

restriction digests. This seed contamination occurred at a very low level (<0.01%) and is reasonable to have occurred due to the proximity of the maternal plants in the field study (0.5 m). Other putative hybrids were rejected because it was determined that they had the maternal species genotype and had survived the glyphosate treatment without having the physiological resistance trait. This is also reasonable to expect due to the large number of progeny screened and the relatively low glyphosate rate used.

A. spinosus had the highest and most frequent hybridization rates when pollinated by *A. palmeri*, averaging <0.01% in 2006 and 0.4% in 2007 (Table 3 - 2). Seven of eight *A. spinosus* plants in 2007 and two of eight in 2006 had resistant hybrids, indicating that pollen movement and successful fertilization can occur at 3 m distance (Table 3 - 3). This inter-specific hybrid has not previously been reported, but the potential for this hybridization was predicted by Wassom and Tranel (2005). All tested hybrids with *A. spinosus* were confirmed as having increased copy number and ITS alleles from both parents (Table 3 - 3 and Figure 3 - 1).

Glyphosate resistant hybrids were confirmed with *A. hybridus* at a rate of <0.01% and in two *A. tuberculatus* accessions at 0.08% and 0.19% (Table 3 - 2). All of these hybrids occurred in 2007, with four of 16 *A. tuberculatus* plants and two of eight *A. hybridus* plants having resistant hybrids, and hybridization occurring at 1 and 3 m distance (Table 3 - 3). Hybrids with *A. hybridus* were confirmed using EPSPS copy number (Table 3 - 3) and ITS alleles (Figure 3 - 1), while hybrids with *A. tuberculatus* were confirmed using copy number (Table 3 - 3) and ALS alleles (Figure 3 - 2). Six glyphosate resistant hybrids with *A. tuberculatus* were confirmed. Four non-resistant hybrids with *A. tuberculatus* were also confirmed. These plants survived the glyphosate

treatment but were susceptible when measured with an in-vivo leaf disc shikimate assay (Shaner et al. 2005) and they did not have increased EPSPS copy number. This indicates that the *A. palmeri* pollen source was not entirely homozygous for increased copy number and non-resistant hybrids may have occurred in other species that were not detected with the glyphosate screen.

Progeny from confirmed hybrids had variable results. Research into F_2 progeny from a susceptible by resistant *A. palmeri* cross (Chapter 1) indicated that at the screening rate used in this study, about 80% of F_2 progeny survived. Hybrids between *A. spinosus* females and *A. palmeri* males were self-pollinated and produced seeds had survival rates ranging from 50 to 90% in their progeny (Table 3 - 3). Hybrids with *A. tuberculatus* and *A. hybridus* did not produce any seed. This likely indicates reduced fertility in *A. palmeri* hybrids with these two species. The reduced fertility of *A. tuberculatus* by *A. palmeri* hybrids has been reported previously (Franssen et al. 2001; Steinau et al. 2003; Trucco et al. 2007; Wetzel et al. 1999b).

Within the *Amaranthus* genus, dioecy is epistatic to monoecy (Murray 1940). However, the confirmed hybrids between *A. spinosus* and *A. palmeri* were variable. Some were dioecious (only males were observed) and some were monoecious (Table 3 - 3). This cross has not been examined before, and the results indicate that these inter-specific hybrids may be segregating for sex determination genes. Hybrids with *A. tuberculatus* and *A. hybridus* were dioecious (Table 3 - 3).

Hand Crosses

In a small number of hand crosses with *A. palmeri* as the female, putative hybrids were detected with *A. spinosus* and *A. powellii* ssp. *powellii* as the male parent, but not

with other species (data not shown). Using herbicide resistance as a marker for hybridization is difficult in a partially apomictic species, and the primary interest of this study is with *A. palmeri* as a male pollen donor. Inter-specific hybridization may occur between *A. palmeri* as a female and the other five species, but results reported here emphasize *A. palmeri* as a male pollen donor.

Hybridization was detected in a hand cross between maternal *A. spinosus* and paternal *A. palmeri* (Table 3 - 4) at a rate of 1.4%. When using the earlier emergence of stigmas to make crosses to chiefly self-pollinating *Amaranthus* species, 25% self-pollination was still observed (Murray 1940). The small sample size in this study prevents concluding that *A. palmeri* will not pollinate the other species, but it does indicate that the cross occurs less easily than the cross with *A. spinosus*. Hybrids from the cross with *A. spinosus* were confirmed with EPSPS copy number and ITS alleles (Table 3 - 5) and showed the same variation for flowering morphology observed in field study hybrids (Tables 3 - 3 and 5), with both dioecious and monoecious individuals.

Previous studies on hybridization between *A. palmeri* and *A. tuberculatus* yielded varying conclusions. Recently, Trucco et al. (2007) established that inter-specific hybrids with intermediate DNA content had developmental problems and died before flowering or did not produce flowers. Some hybrids were triploid, indicating the contribution of an unreduced male gamete. Additionally, the occurrence of non-hybrid progeny from maternal *A. palmeri* plants indicated the presence of unreduced female gametes resulting in apomixis. The only fertile bridge for gene transfer was the triploid hybrid. Other studies [e.g. (Franssen et al. 2001)] have demonstrated hybridization between *A. palmeri* and *A. tuberculatus*, using herbicide resistance as a marker, and transfer of resistance

from an *A. tuberculatus* source to *A. palmeri* through one backcross has been shown (Wetzel et al. 1999b). Taken together, these studies suggest that gene flow between these two species can occur, but at low levels, and may involve complex meiotic processes.

The most robust trend for inter-specific hybridization and gene transfer from *A. palmeri* is with *A. spinosus*. Hybridization with *A. hybridus* and *A. tuberculatus* occurred on a much less frequent basis. Based on the sample size, hybridization with *A. spinosus* occurs at a high enough rate to be a significant management and ecological concern for transfer of glyphosate resistance. Because the experimental design used glyphosate resistance as a screening marker, the hybridization rates reported here are likely lower than actual hybridization. Hybrids may have formed that did not inherit the resistance trait. These rates do indicate the frequency with which the first step for glyphosate resistance introgression into related *Amaranthus* species could occur in the field.

REFERENCES

Culpepper, A. S., T. L. Grey, W. K. Vencill, J. M. Kichler, T. M. Webster, S. M. Brown, A. C. York, J. W. Davis, and W. W. Hanna. 2006. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci. 54:620-626.

Franssen, A. S., D. Z. Skinner, K. Al-Khatib, M. J. Horak, and P. A. Kulakow. 2001. Interspecific hybridization and gene flow of ALS resistance in *Amaranthus* species. Weed Sci. 49:598-606.

Grant, W. F. 1959a. Cytogenetic studies in *Amaranthus*. II. Natural interspecific hybridization between *A. dubius* and *A. spinosus*. Can. J. Botany 37:1063-1070.

Grant, W. F. 1959b. Cytogenetic studies in *Amaranthus*. I. Cytological aspects of sex determination in dioecious species. Can. J. Botany 37:413-417.

Grant, W. F. 1959c. Cytogenetic studies in *Amaranthus*. III. Chromosome numbers and phylogenetic aspects. Can. J. Genet. Cytol. 1:313-328.

Greizerstein, E. J. and L. Poggio. 1995. Meiotic studies of spontaneous hybrids of *Amaranthus*: genome analysis. Plt. Breed. 114:448-450.

Heap, I. 2008. The International Survey of Herbicide Resistant Weeds. Online. Available at www.weedscience.com (accessed October 21, 2008).

Hidayat, I., J. Baker, and C. Preston. 2006. Pollen-mediated gene flow between paraquat-resistant and susceptible hare barley (*Hordeum leporinum*). Weed Sci. 54:685-689.

Murray, M. G. and W. F. Thompson. 1980. Rapid isolation of high molecular-weight plant DNA. Nuc. Acids Res. 8:4321-4325.

Murray, M. J. 1940. The genetics of sex determination in the family *Amaranthaceae.* Genetics 25:409-431.

Norsworthy, J. K., G. M. Griffith, R. C. Scott, K. L. Smith, and L. R. Oliver. 2008. Confirmation and control of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) in Arkansas. Weed Technol. 22:108-113.

Rayburn, A. L., R. McCloskey, T. C. Tatum, G. A. Bollero, M. R. Jeschke, and P. J. Tranel. 2005. Genome size analysis of weedy Amaranthus species. Crop Sci. 45:2557-2562.

SAS Institute Inc. 2004. SAS OnlineDoc® 9.1.3. SAS Institute Inc., Cary, NC.

Sauer, J. D. 1950. The grain amaranths: A survey of their history and classification. Annal. Miss. Bot. Garden 37:561-632.

Shaner, D. L., T. Nadler-Hassar, W. B. Henry, and C. H. Koger. 2005. A rapid in vivo shikimate accumulation assay with excised leaf discs. Weed Sci. 53:769-774.

Steckel, L. E., C. L. Main, A. T. Ellis, and T. C. Mueller. 2008. Palmer amaranth (*Amaranthus palmeri*) in Tennessee has low level glyphosate resistance. Weed Technol. 22:119-123.

Steinau, A. N., D. Z. Skinner, and M. Steinau. 2003. Mechanism of extreme genetic recombination in weedy *Amaranthus* hybrids. Weed Sci. 51:696-701.

Trucco, F., M. R. Jeschke, A. L. Rayburn, and P. J. Tranel. 2005a. Promiscuity in weedy amaranths: High frequency of female tall waterhemp (*Amaranthus tuberculatus*) x smooth pigweed (*A. hybridus*) hybridization under field conditions. Weed Sci. 53:46-54.

Trucco, F., M. R. Jeschke, A. L. Rayburn, and P. J. Tranel. 2005b. *Amaranthus hybridus* can be pollinated frequently by *A. tuberculatus* under field conditions. Heredity 94:64-70.

Trucco, F., D. Zheng, A. J. Woodyard, J. R. Walter, T. C. Tatum, A. L. Rayburn, and P. J. Tranel. 2007. Nonhybrid progeny from crosses of dioecious amaranths: Implications for gene-flow research. Weed Sci. 55:119-122.

Vencill, W. K., T. L. Grey, A. S. Culpepper, T. A. Gaines, and P. Westra. 2008. Herbicide-resistance in the Amaranthaceae. J. Plant Dis. Protect. Special Issue XXI:41-44.

Wassom, J. J. and P. J. Tranel. 2005. Amplified fragment length polymorphism-based genetic relationships among weedy Amaranthus species. J. Heredity 96:410-416.

Wetzel, D. K., M. J. Horak, and D. Z. Skinner. 1999a. Use of PCR-based molecular markers to identity weedy *Amaranthus* species. Weed Sci. 47:518-523.

Wetzel, D. K., M. J. Horak, D. Z. Skinner, and P. A. Kulakow. 1999b. Transferal of herbicide resistance traits from *Amaranthus palmeri* to *Amaranthus rudis*. Weed Sci. 47:538-543.

TABLES

Table 3 - 1. Origin and United States Department of Agriculture Agriculture Research Service National Plant Germplasm System plant introduction (USDA-ARS NPGS PI) numbers of species used in hybridization studies and their origin. Plant height and total seed production from *Amaranthus* species grown in the field in Colorado in 2006 and 2007.

			2006			2007		
Species	USDA-ARS NPGS PI	Common name, origin	Plant Height (cm)[1]	Total Seeds		Plant Height (cm)[1]	Total Seeds	
1-*A. palmeri*	--	Palmer amaranth, GA	156 AB	44,243	BCD	71 ABC	9,757	D
2-*A. palmeri*	632235	Palmer amaranth, AZ	165 A	6,448	D	67 BC	13,687	D
3-*A. hybridus*	--	Smooth pigweed, KS	128 CD	159,470	A	79 AB	82,089	AB
4-*A. hybridus*	603886	Smooth pigweed, OH	129 CD	66,335	B	85 A	91,666	A
5-*A. powellii* ssp. *bouchonii*	595317	Powell amaranth, CA	107 DE	3,696	D	68 BC	14,882	D
6-*A. powellii* ssp. *powellii*	604671	Powell amaranth, WA	137 BC	34,274	BCD	56 C	13,146	D
7-*A. retroflexus*	572263	Redroot pigweed, IA	136 BC	56,109	BC	73 AB	49,936	BCD
8-*A. spinosus*	482057	Spiny amaranth, Zimbabwe	103 E	48,132	BCD	n/a	n/a	
9-*A. spinosus*	612848	Spiny amaranth, Indonesia	n/a[2]	n/a		70 ABC	71,856	ABC
10-*A. tuberculatus*	553806	Waterhemp, IA	n/a	n/a		53 C	40,141	CD
11-*A. tuberculatus*	603873	Waterhemp, NE	181 A	8,464	CD	72 AB	32,510	D
12-*A. tuberculatus*	--	Waterhemp, KS	183 A	153,055	A	n/a	n/a	

1 – Means within a column with the same letter are not significantly different ($P<0.05$).

2 – Individual accession not included.

Table 3 - 2. Frequency of putative and confirmed glyphosate resistant hybrids that occurred under field pollination conditions from male *Amaranthus palmeri* pollen donor to other *Amaranthus* species in 2006 and 2007.

Species	No. Maternal Plants Screened[1]	Total No. Emerged	N	Mean % Emergence[2] ± SE	Total No. Surviving Glyphosate Treatment	Mean % Survival ± SE	Total No. Confirmed Resistant Hybrids[3]	Mean % Hybrids ± SE
1-*A. palmeri*	9	21,800	76	36 ± 6	6,977	27.90 ± 2.80	1/1	--
2-*A. palmeri*	8	3,700	64	32 ± 6	662	21.10 ± 2.46	--	--
3-*A. hybridus*	16	91,450	154	40 ± 3	8	0.01 ± 0.01	2	0.008 ± 0.006
4-*A. hybridus*	12	52,900	106	41 ± 4	1	0.01 ± 0.01	0	--
5-*A. powellii* ssp. *bouchonii*	12	8,700	85	20 ± 2	0	0.00 ± 0.00	0	--
6-*A. powellii* ssp. *powellii*	16	44,150	142	24 ± 2	17	0.11 ± 0.04	0	--
7-*A. retroflexus*	16	65,200	140	39 ± 4	7	0.54 ± 0.30	0	--
8-*A. spinosus*	8	60,400	118	96 ± 9	2	0.005 ± 0.004	2	0.005 ± 0.004
9-*A. spinosus*	8	42,350	76	27 ± 2	195	0.43 ± 0.07	3/3	0.43 ± 0.07
10-*A. tuberculatus*	8	3,350	62	3 ± 1	3	0.24 ± 0.17	2	0.079 ± 0.059
11-*A. tuberculatus*	11	3,400	95	8 ± 2	7	0.35 ± 0.25	4	0.19 ± 0.13
12-*A. tuberculatus*	3	5,550	30	17 ± 4	0	0.00 ± 0.00	0	--

1 - Combined data from 2006 and 2007 field studies

2 - % emergence calculated based on total seed number estimates

3 - Reasons for rejecting putative hybrids included confirmation that they were Palmer amaranth seed contaminants or that they were plants of the maternal species that survived the glyphosate treatment

Table 3 - 3. Data used to confirm hybrids between *Amaranthus palmeri* and related *Amaranthus* species, including 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative copy number, internal transcribed spacer (ITS) restriction polymorphisms, acetolactate synthase (ALS) restriction polymorphisms, flowering phenotype, and resistance segregation in F_2 progeny. EPSPS copy number and ITS and ALS restriction profiles of parental species are provided for reference.

				----------Confirmed Hybrid Results----------					
Species	Year	Maternal Plant ID[1]	EPSPS Copy No.[2]	-----ITS Restriction-----[3]			ALS Restriction[4]	Flowering Phenotype	Mean % Resistant Progeny ± SE[5]
				BsaAI	HaeII	XhoI			
1-*A. palmeri*	--	--	1.1	+	-	-	1, 2		
	2007	57-4	64.0	+	-	-	1, 2		
3-*A. hybridus*	--	--	0.9	-	+				
	2007	20-2	104.0	+/-	+/-			dioecious male	no seed
	2007	63-4	67.0	+/-	+/-			dioecious female	no seed
8-*A. spinosus*	--	--	1.0			+			
	2006	26-2	64.0			+/-		monoecious	seed not tested
	2006	37-3	22.1			+/-		dioecious male	no seed

continued next page

Table 3 – 3 continued

9-*A. spinosus*	--	--	0.9	+			
	2007	65-4	40.3	+/-		monoecious	70 ± 16
	2007	65-4	48.5	+/-		monoecious	seed not tested
	2007	65-4	19.2	+/-		dioecious male	no seed
	2007	5-1				monoecious	61 ± 15
	2007	23-2				monoecious	94 ± 4
	2007	26-2				monoecious	50 ± 0
	2007	47-3				monoecious	95 ± 3
	2007	52-3				monoecious	92 ± 8
	2007	72-4				monoecious	48 ± 16
10-*A. tuberculatus*	--	--	0.8		2, 3		
	2007	38-3	99.3		1, 2, 3	dioecious male	no seed
	2007	38-3	58.4		1, 2, 3	dioecious male	no seed
11-*A. tuberculatus*	--	--	1.0		2, 3		
	2007	36-2	78.8		1, 2, 3	dioecious male	no seed
	2007	41-3	58.4		1, 2, 3	dioecious female	no seed
	2007	56-4	122.2		1, 2, 3	dioecious male	no seed
	2007	56-4	70.2		1, 2, 3	dioecious male	no seed

1 – Individual ID number and row number for maternal plant from which confirmed hybrid originated. Rows 2 and 3 were 1 m from pollen source, and rows 1 and 4 were 3 m from pollen source.

2 - EPSPS copy number measured relative to ALS copy number using quantitative PCR on genomic DNA

3 - ITS protocol following Wetzel et al. (1999); parental species results were confirmed on these accessions

4 – ALS restriction polymorphism with bands numbered 1, 2, and 3 following the methods of Trucco et al. (2007); parental species results were confirmed on these accessions.

5 - If confirmed hybrid produced seed through self-pollination, mean of three replications following 0.4 kg ha^{-1} glyphosate application

Table 3 - 4. Hybridization in hand crosses using *Amaranthus palmeri* as a pollen donor and related *Amaranthus* species as pollen recipients and glyphosate resistance as a screening marker for hybrids.

Species	Total No. Seeds Screened	Total Plants Emerged	Mean % Emergence[1]	Total No. Survivors	Mean % Survival	Total No. Confirmed Hybrids[2]	Mean % Hybrids
3-*A. hybridus*	8970	462	3.20	1	1.59	0/1	0.00
4-*A. hybridus*	9384	12	0.25	0	0.00	0	0.00
5-*A. powellii* ssp. *bouchonii*		2	--	1	50.00	0/1	0.00
6-*A. powellii* ssp. *powellii*	9270	33	0.36	0	0.00	0	0.00
7-*A. retroflexus*		27	--	0	0.00	0	0.00
9-*A. spinosus*	6708	533	8.80	10	1.44	4/4	1.44
10-*A. tuberculatus*	4470	1	0.02	0	0.00	0	0.00
11-*A. tuberculatus*	7050	1	0.01	0	0.00	0	0.00

1 - % emergence calculated based on total seed number estimates

2 - Reasons for rejecting putative hybrids included confirmation that they were Palmer amaranth seed contaminants or that they were plants of the maternal species that survived the glyphosate treatment

Table 3 - 5. Data used to confirm hybrids from hand crosses using glyphosate resistant *Amaranthus palmeri* pollen applied to *A. spinosus*, including 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) relative copy number, internal transcribed spacer (ITS) restriction polymorphisms, flowering phenotype, and resistance segregation in progeny. EPSPS copy number and ITS restriction profiles of parental species are provided for reference.

		----------Confirmed Hybrid Results----------		
ID	EPSPS Copy No.[1]	ITS Restriction[2]	Flowering Phenotype	Mean % Resistant Progeny ± SE[3]
		XhoI		
A. palmeri	1.1	-		
A. spinosus	0.9	+		
Hybrid 1	29	+/-	monoecious dioecious	78 ± 22
Hybrid 2	34	+/-	male	no seeds
Hybrid 3	77	+/-	dioecious male	no seeds
Hybrid 4	67	+/-	monoecious	83 ± 17

1 - EPSPS copy number measured relative to acetolactate synthase (ALS) copy number using quantitative PCR on genomic DNA

2 - ITS protocol following Wetzel et al. (1999); parental species results were confirmed on these accessions

3 - If confirmed hybrid produced seed through self-pollination, mean of three replications following 0.4 kg ha^{-1} glyphosate application

FIGURES

Figure 3 - 1. Analysis of internal transcribed spacer (ITS) restriction polymorphisms used to confirm putative glyphosate resistant hybrids. 1-4: *Amaranthus palmeri* ITS band undigested, BsaAI, HaeII, and XhoI. 5-7: *A. hybridus* ITS band undigested, BsaAI, and HaeII. 8-10: *A. hybridus* by *A. palmeri* glyphosate resistant hybrid ITS band undigested, BsaAI, and HaeII. 11-12: *A. spinosus* ITS band undigested and XhoI. 13-14: *A. spinosus* by *A. palmeri* glyphosate resistant hybrids ITS band undigested and XhoI.

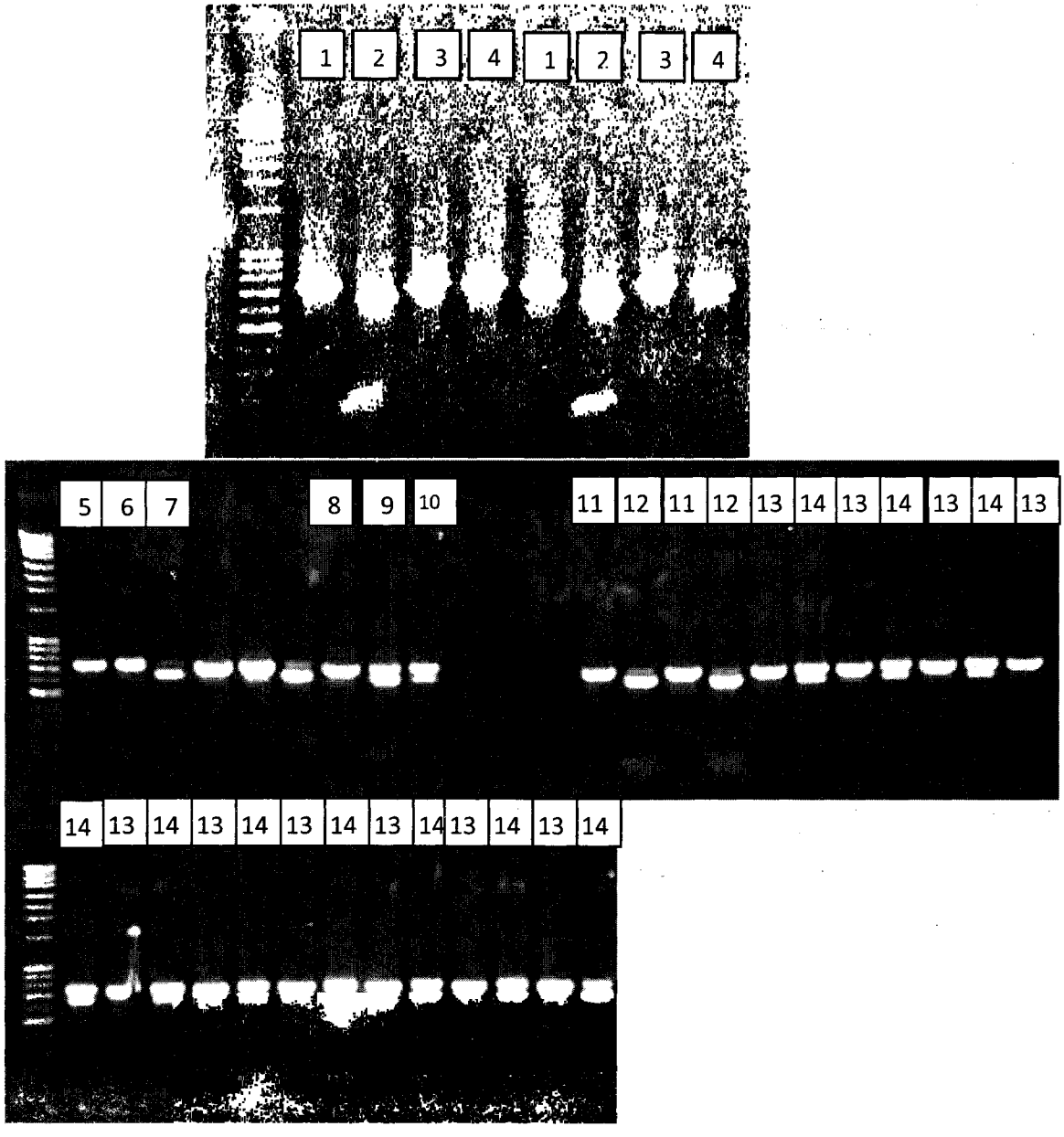


Figure 3 - 2. Analysis of EcoRV restriction site polymorphism in *Amaranthus tuberculatus*, *A. palmeri* (upper panel), and glyphosate resistant *A. tuberculatus* by *A. palmeri* F_1 hybrid plants (lower panel). The acetolactate synthase (ALS) gene was amplified from each template using primers ALSF2 and ALSR1, then digested with EcoRV for 2 h. Both digested and undigested aliquots were analyzed on agarose gel. The bands used to identify *A. tuberculatus* (2 and 3) and *A. palmeri* (1 and 2) are identified.