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CONSTRAINTS ON THE EVOLUTION OF TOLERANCE TO HERBICIDE IN THE COMMON MORNING GLORY: RESISTANCE AND TOLERANCE ARE MUTUALLY EXCLUSIVE

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Evolutionary biologists explain the maintenance of intermediate levels of defense in plant populations as being due to trade-offs, or negative genetic covariances among ecologically important traits. Attempts at detecting trade-offs as constraints on the evolution of defense have not always been successful, leading some to conclude that such trade-offs rarely explain current levels of defense in the population. Using the agricultural pest *Ipomoea purpurea*, we measured correlations between traits involved in defense to glyphosate, the active ingredient in Roundup, a widely used herbicide. We found significant allocation costs of tolerance, as well as trade-offs between resistance and two measures of tolerance to glyphosate. Selection on resistance and tolerance exhibited differing patterns: tolerance to leaf damage was under negative directional selection, whereas resistance was under positive directional selection. The joint pattern of selection on resistance and tolerance to leaf damage indicated the presence of alternate peaks in the fitness landscape such that a combination of either high tolerance and low resistance, or high resistance and low tolerance was favored. The widespread use of this herbicide suggests that it is likely an important selective agent on weed populations. Understanding the evolutionary dynamics of herbicide defense traits is thus of increasing importance in the context of human-mediated evolution.

KEY WORDS: **Agro-ecosystem, fitness costs, glyphosate, selection, trade-offs.**

A central goal in evolutionary biology is to understand the forces that create and preserve variation in ecologically important traits. A general class of ecologically relevant traits are those that act in a defensive capacity to maintain the fitness of an organism attacked by predators, herbivores or pathogens. It is generally assumed that, given attack, selection will move the mean level of defense in a population to the point where all individuals are defended, with the result being that maximal levels of defense are attained and genetic variation for defense depleted (Rausher and Simms 1989).

However, considerable genetic variation for defensive traits has been documented in multiple biological systems and levels of defense are often at intermediate values (Berenbaum et al. 1986; Simms and Rausher 1987, 1989; Fineblum and Rausher 1995; Tiffin and Rausher 1999; Stinchcombe and Rausher 2001, 2002; Baucom and Mauricio 2004). Furthermore, researchers have found that levels of host plant defense increase in the presence of natural enemies, such that the level of plant defense often "matches" the chemical response phenotype of the herbivore (Berenbaum and Zangerl 1998, 2006). To explain these findings, theoretical models have shown that the existence of trade-offs, or genetic constraints between traits, can act to maintain intermediate levels of defensive traits in the population

(Simms and Rausher 1987; Fineblum and Rausher 1995; Mauricio 1998).

These predictions have been tested by researchers assessing the potential for trade-offs between two important defense adaptations: resistance and tolerance. This question has received attention because a key assumption of the prediction that trade-offs between resistance and tolerance maintain intermediate levels of defense is that the two traits serve redundant purposes and incur fitness costs (Mauricio et al. 1997). Thus, having both defensive strategies leads to a cost greater than would be experienced if only one strategy was employed, with the result being that selection should move the population to either a highly resistant or highly tolerant state, but not both. However, only a few investigations provide evidence for a trade-off between resistance and tolerance (Fineblum and Rausher 1995; Stowe 1998; Pilson 2000; Fornoni et al. 2003).

A recent meta analysis and a synthetic review of the agricultural and ecological literature found little support for the presence of trade-offs between resistance and tolerance, suggesting that a pattern of simultaneous allocation to both defense strategies should be common in natural populations (Leimu and Koricheva 2006; Núñez-Farfán et al. 2007). As an alternative to trade-offs between resistance and tolerance, stabilizing selection, due to a nonlinear cost or benefit function of allocation to defense, has been proposed to explain the presence of intermediate values of defensive traits (Tiffin and Rausher 1999; Fornoni et al. 2004a). Evidence for a nonlinear cost or benefit function is mixed, with some studies uncovering their presence (Mauricio et al. 1997; Pilson 2000; Fornoni et al. 2004b) and others failing to detect a nonlinear component (Tiffin and Rausher 1999). Further, models that explicitly consider defense trade-offs in the form of allocation or fitness costs invoke this as another mechanism by which intermediate levels of defensive traits could be maintained in a population (Simms and Rausher 1987; Rausher and Simms 1989; Fineblum and Rausher 1995). In contrast to the few studies that have shown a trade-off between resistance and tolerance, fitness costs of defense are widely empirically supported (Núñez-Farfán et al. 2007).

Like herbivores and pathogens, the anthropogenic application of herbicides can significantly reduce plant fitness. Likewise, we can use similar methodology to understand the evolution of defense against the action of herbicides. We have previously applied the cost/benefit framework of Simms and Rausher (1987) to the potential for constraints on tolerance to herbicide in the common morning glory, *Ipomoea purpurea*. Specifically, we have documented the presence of genetic variation for tolerance to glyphosate, found evidence of a fitness cost of tolerance, and examined how tolerance varies across a wide geographical scale (Baucom and Mauricio 2004, 2008). However, the relationship between tolerance and resistance to glyphosate has yet to be examined. Here, we investigate the relationship between resistance and tolerance to glyphosate, the active ingredient in Roundup (Monsanto, St. Louis, MO), a herbicide used in agroecosystems around the world.

An evolutionary analysis of herbicide defense has potential significance in the applied sciences, as a weed could evade the control of herbicides by either resistance or tolerance. Should a weed possess both traits, the evolutionary trajectory of either trait could be affected if selection for or opposing one indirectly selects for the other. Further, should a weed employ both strategies, the two traits will act epistatically of one another. For example, tolerance will not be expressed if a plant is completely resistant and thus does not experience damage. Alternatively, plants that exhibit high tolerance will experience little selection for resistance because damage to the plant will not significantly reduce fitness (Simms and Triplett 1994). Selection studies have been proposed as a means of disentangling the two traits to understand their relative contributions to plant fitness (Stowe 1998; Stowe et al. 2000), and although this has also been done in the context of understanding how tolerance affects the evolution of herbivore populations (Espinosa and Fornoni 2006), it is a methodology that has otherwise rarely been explored.

Another tactic used by researchers is to study traits correlated with resistance (Mauricio and Rausher 1997) or tolerance (Juenger and Bergelson 2000; Tiffin 2000; Weinig et al. 2003) in an attempt to discern their mechanistic basis. However, there are many traits potentially responsible for either form of defense, and for this reason researchers have chosen to "operationalize" estimates of both characters. Resistance is estimated as "1 − the proportion damage" (Simms and Rausher 1987), whereas tolerance is estimated as the relationship between fitness and damage for genetically related individuals (Simms and Triplett 1994; Fineblum and Rausher 1995).

The operational estimate of resistance from the herbivory literature is analogous to the measure of herbicide resistance from the weed science literature. In describing herbicide resistance, researchers use a broad range of phenotypic assays, including injury ratings, measures of the proportion of the plant that exhibited damage symptoms, and changes in biomass given herbicide application (Dekker and Duke 1995). As done in both the herbicide resistance and herbivory literature, we quantify resistance by measuring apparent vegetative damage. Our operational definition of tolerance comes from the herbivory literature, and we measure this trait as the fitness response of plants following the application of herbicide.

The specific objectives of this study were to investigate the potential for constraints on the evolution of tolerance to glyphosate in the common morning glory, *I. purpurea* (L.) Roth. We assessed the potential for fitness or allocation costs of tolerance and the potential for negative genetic correlations between

tolerance and resistance. We asked the following questions: (1) What is the joint pattern and type of selection acting on tolerance and resistance to glyphosate? (2) Are tolerance and resistance negatively genetically correlated? Previously, we have documented the presence of fitness costs associated with tolerance to glyphosate among selfed, field-collected maternal lines (Baucom and Mauricio 2004). Because the existence of fitness costs is assumed by the models attempting to explain constraints on the evolution of defense, we again asked if (3) tolerance has fitness costs, this time using family lines generated from a crossing scheme designed to remove potential maternal effects.

Materials and Methods

EXPERIMENTAL SYSTEM

The common morning glory, *I. purpurea* (L.) Roth. (Convolvulaceae), is a weedy annual vine that grows in disturbed habitats throughout the southeastern United States. In Georgia, plants germinate from mid-May to late August; flowering typically occurs about six weeks after germination and continues until the plants begin to senesce or are killed by the frost, typically at the end of November or the beginning of December. Individual flowers open for a single morning, and plants bear multiple flowers daily (approximate range: 0–55). Flowers are pollinated almost exclusively by bumblebees (Ennos 1981), although this species is also capable of self-fertilization. Fruits mature four to six weeks after pollination and produce from one to six seeds each. The average outcrossing rate for this species has been estimated as 70% in natural populations (Ennos 1981; Brown and Clegg 1984).

In the southeastern United States, *I. purpurea* is often found in maize, soy, and cotton fields. Previous work has shown that a population of *I. purpurea* collected from an agricultural field in Oconee County, Georgia was tolerant to glyphosate, the main ingredient in the herbicide Roundup, and that there was genetic variation underlying this tolerance (Baucom and Mauricio 2004). The plants in this study and in greenhouse studies (R. S. Baucom, pers. obs.) have shown similar patterns of damage after application of the herbicide in that sprayed individuals exhibit significant damage to leaf and other vegetative tissue yet are able to regrow, produce flowers, and set seed. Specifically, 1.5 weeks after application of the herbicide, the leaves begin to exhibit yellowing and necrosis. On many plants, the leaves and the apical meristem completely die, rendering the plant stunted or dead. After being sprayed, plants that survive and produce flowers appear to do so from new stem growth (R. S. Baucom, pers. obs.).

Glyphosate is a nonspecific postemergence herbicide (Grossbard and Atkinson 1985), which enters the plant by diffusion and is mobile throughout the phloem (Caseley and Coupland 1985). Glyphosate accumulates in the apical meristems and other sites of sugar utilization (Franz et al. 1997), and causes plant death by inhibiting the biosynthesis of aromatic amino acids (Amrhein et al. 1980; Steinrucken and Amrhein 1980) by inhibiting 5-enol-pyruvylshikimate-3-phosphate synthase, a key enzyme in the shikimate pathway. This reaction is a competitive one and occurs in the cytosol of the chloroplast, thus the potential exists that tolerance or resistance could be governed by cytoplasmic factors inherited through the maternal line.

EXPERIMENTAL DESIGN

Assessment of natural variation

In the fall of 2000, seeds were randomly collected from a total of 122 individuals growing on the University of Georgia's Plant Sciences Farm ($N = 101$; Oconee Co.) and from a second field located > 12 km away ($N = 21$). Because *I. purpurea* employs a mixed-mating system, seeds collected from each plant are considered half-sibling progeny from a maternal line. In the greenhouse, we randomly planted five replicate seeds from each maternal line within four glyphosate treatments, applied on a kilogram acid equivalent (a.e.) per hectare basis: 1.121 kg a.e. ha^{-1}, 0.56 kg a.e. ha^{-1}, 0.28 kg a.e. ha^{-1}, and 0 kg a.e. ha^{-1} as part of an initial investigation designed to assess the level of variation in response to being sprayed by glyphosate. The height of each plant was measured immediately before the application of glyphosate, and mortality and height of each plant was recorded one month after glyphosate application. Maternal lines exhibiting either the least reduction in height or the greatest reduction in height when treated with 1.121 kg a.e. ha^{-1} of glyphosate (the manufacturer's recommended field dose) were considered as either the "least susceptible" or "most susceptible" lines, respectively (Fig. 1).

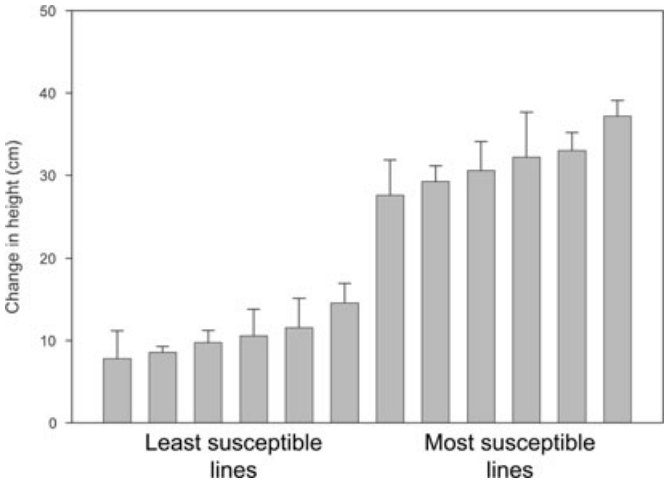

Figure 1. Average reduction in height (cm) of each line used to generate experimental individuals. The six lines exhibiting the least reduction in height after glyphosate application were considered the "least susceptible" lines whereas the six lines showing the greatest reduction in height were considered the "most susceptible."

Maternal lines that exhibited high mortality were also invariably the lines that exhibited the greatest reduction in height, yet no maternal lines exhibited complete mortality. Thus, mortality did not affect our ability to select the highly susceptible lines.

To generate experimental individuals, field-collected replicate seeds from 12 maternal lines were used as parents in a full-sibling breeding design. Six individuals from the lines considered "least susceptible" were reciprocally crossed among themselves in one diallel, as were six individuals from lines considered "most susceptible." Due to limitations of space, the diallels could not be replicated, thereby confounding effects of the diallel with susceptibility level. To control for this, the timing of crosses between plants within a diallel was made in a random order such that neither diallel was exclusively pollinated at any specific time. Further, we chose to use field-collected replicate sibling seeds as parents in the crossing design rather than continuing to select the lines for either increased or decreased susceptibility in an attempt to capture the current level of natural variation for response to glyphosate application in *I. purpurea*. Use of the "sibling selection" scheme is similar to choosing entire families as the unit of selection, with the difference that the selected individuals have not contributed to the estimate of their family mean (Falconer and Mackay 1996). Ten of the parents used in the crosses originated from the Plant Sciences Farm (Watkinsville, GA), whereas the other two were collected from a field located > 12 km away from this site.

For crosses, a single seed from each of the 12 field-collected maternal lines was grown in a 12-inch pot and fertilized every other week with a 10–30–20 fertilizer (Peter's Blossom Booster, J. R. Peters, Inc., Allentown, PA) in the greenhouse. Once individuals began flowering, reciprocal pollinations were performed among all individuals within a diallel by removing an anther from a pollen parent and touching it to the stigma of the seed parent. Anthers were removed from each pollen donor and pollen recipient the night before crosses were made to prevent self-pollination. These crosses produced 60 full-sibling families within 12 maternal and 12 paternal half-sibling families.

Field protocol

On 9 July 2004 we planted 140 seeds from each of the paternal half-sibling families (28 replicates of each full-sibling family, including reciprocals, for a total of 1,680 seeds) in a randomized split-plot design with treatment plots nested within two spatial blocks. Treatment with herbicide was the whole-plot factor with genetic family as the subplot factor. Seven replicates per full-sibling family were randomly planted within treatment plots of each block. This experiment was planted in a twice-plowed and disked agricultural field on the University of Georgia's Plant Sciences Farm. To ensure germination we nicked each seed prior to planting. Within each plot we planted seeds in a grid with 1-m^2 separating each experimental individual, and marked planted seeds with straws to facilitate finding experimental plants. We removed the vegetation within 0.3 m around experimental individuals once over the course of the experiment to deter herbivory from the cotton rat, *Sigmodon hispidus*, but otherwise let competitive weeds grow undeterred. Each plant was allowed to grow up a 1-m tall bamboo stake that mimics *I. purpurea* growth in agricultural fields and allows for easy identification of experimental plants. Seedlings began emerging within one week of being planted, and plants began flowering by August 31 in the control plots. Fruits began maturing by 18 September. We applied glyphosate at a rate of 1.121 kg a.e. ha^{-1} with a pressurized CO_2 plot sprayer, which keeps droplet size and spray intensity constant (R & D Sprayers, Opelousas, LA), on August 15, 2004 to experimental plants within the glyphosate treatment plots of each block.

Data collection

On 9 September, approximately three weeks following glyphosate application, we collected mortality data of both sprayed and control individuals. No control plants died during this time. We also counted the total number of leaves that remained on each sprayed plant as well as the number of leaves exhibiting symptoms of glyphosate damage for an estimate of the proportion of plant that was damaged. After fruits began maturing, we collected seeds during four rounds of collection until all plants were killed by frost on 4 December. Viable seeds were counted, and the number of seeds produced by each plant was used as an estimate of fitness. Nonviable morning glory seeds are shrivelled in appearance, and are easily separated from viable seeds. Only individuals that survived to glyphosate application were included in the analysis; those that survived to the application of glyphosate but died as a result of its application or did not produce seed were given a fitness score of "0."

DATA ANALYSIS

Genetic variation for resistance and tolerance

The presence of additive genetic variation for resistance was determined by assessing if the proportion of the plant damaged from glyphosate, estimated by dividing the number of leaves exhibiting yellowing or necrosis by the total number of leaves remaining on the plant, varied among maternal or paternal half-sibling families using PROC GLM of SAS (version 9.1, SAS, Cary, NC). For this trait, we used only the plants treated with glyphosate. In this analysis, the proportion damage was the response variable with diallel, block, sire and dam nested within diallel, and their interaction as the independent variables. All effects including the block effect (see Simms and Rausher 1989) were considered fixed. The proportion damage was arcsine square-root transformed before analysis.

We measured two types of tolerance: tolerance to glyphosate application and tolerance to leaf damage following glyphosate

application (see section "Operational definitions of resistance and tolerance"). Estimating tolerance to glyphosate necessitates the use of control plants that are not sprayed with glyphosate whereas estimating tolerance to leaf damage does not; because of this we tested for the presence of additive genetic variation differently between the two measures of tolerance. To test for the presence of additive genetic variation for tolerance to glyphosate, the MIXED procedure of SAS was used to conduct an analysis of variance. This analysis used the fitness response of plants in both the control and treatment plots, which required that we use the block and the block by glyphosate treatment interaction terms as the error term to test for the main effect of glyphosate treatment as is suggested for split-plot designs (Snedecor and Cochran 1989; Littell et al. 1996). We tested the significance of the random effects of block and the block by treatment interaction with likelihood-ratio tests, comparing the – 2 Log likelihoods of models with and without each effect in turn (Littell et al. 1996). To test for the presence of additive genetic variation for tolerance to leaf damage, the GLM procedure of SAS was used to conduct analysis of covariance. In this analysis, the fitness of treated plants was the response variable with the proportion damage each individual exhibited as the covariate and diallel, block, sire and dam nested within diallel, and their interaction as the independent variables.

For both analyses, relative fitness was the response variable and was calculated by dividing each fitness value by the average fitness of all individuals. Examination of the data revealed that fitness was nonnormal and exhibited positive skew due to zero values in the dataset. To improve normality and homogenize variances we performed a square-root (y+1) transformation, which both improved normality and reduced heteroskedasticity upon visual examination of the residuals. In the analysis of tolerance to glyphosate, the term(s) of interest were the interactions between sire and treatment; significant interactions indicate that glyphosate application did not affect the fitness of all paternal half-sibling families equally and provides evidence of additive genetic variation for tolerance. Likewise, a significant interaction between sire and the proportion leaf damage indicates that the effect of leaf damage following glyphosate on fitness was not the same among the paternal half-sibling families and is evidence of additive genetic variation for tolerance to leaf damage. The interactions between dam and treatment, or dam by the proportion leaf damage suggest that maternal lines vary in their level of tolerance; this effect is governed by both additive and nonadditive factors. We chose to assess the potential for variation among maternal half-sibling families given that we know very little about the heritability of glyphosate tolerance, and such an effect could be manifest through cytoplasmic factors. When modeling resistance and the two measures of tolerance, dam, sire, or sire by dam effects were considered fixed, given that the lines used in this experiment were chosen specifically based on their family mean response to glyphosate application in the initial greenhouse experiment.

Operational definitions of resistance and tolerance

One resistance measure and two measures of tolerance were estimated for each half-sibling family. Similar to studies assessing resistance to herbivory, we operationally defined resistance as the average of $(1 - p)$ for each half-sibling family where p equaled the proportion vegetative damage a plant displayed following glyphosate application (Simms and Rausher 1987). In the herbivory literature, this method of measuring resistance assumes plants showing little damage do so because a resistance character has functioned to keep them from being damaged. However, another possibility is that the herbivore abundance was low, such that the plants were simply missed by herbivores rather than truly resistant (Mauricio 1998, 2000). Because we sprayed each plant with glyphosate at the same time, and with the same concentration of herbicide, the response of each plant given the application of herbicide should reflect how well the mechanisms of resistance work to detoxify or protect the plant from damage.

As resistance is a measure of the vegetative response to the application of glyphosate, tolerance to glyphosate is estimated by assessing the effect of glyphosate on the plant's fitness. Tolerance was measured in two different ways, and similarly to Simms and Triplett (1994): as the difference in fitness between related individuals that were either sprayed or in a control environment (tolerance to glyphosate, $W_d - W_u$), and as an estimate of fitness in response to the amount of leaf damage sustained (tolerance to leaf damage). It is unknown if these two measures of tolerance are independent; measuring tolerance as $W_d - W_u$ allows us to quantify the effects of damage given two explicitly manipulated levels of damage, whereas estimating tolerance to leaf damage allows us to estimate the fitness response given the amount of damage expressed by each individual, and thus could potentially provide a more "fine-scale" measure of tolerance.

For each family, tolerance to glyphosate application was estimated by subtracting the mean relative fitness of individuals that were not treated with glyphosate from the mean relative fitness of individuals from the same half-sibling family, but sprayed with glyphosate (Tiffin and Rausher 1999; Weinig et al. 2003; Baucom and Mauricio 2004). Tolerance to leaf damage was estimated by performing a regression of untransformed relative fitness onto proportion leaf damage separately for each half-sibling family (Simms and Triplett 1994; Mauricio et al. 1997; Tiffin and Rausher 1999), which is similar to defining tolerance as a norm of reaction in response to an environmental gradient of increasing damage following spray (Abrahamson and Weis 1997). Both operational estimates of tolerance were made using the residuals of untransformed relative fitness values after the effects of the block were removed.

Preliminary analysis of tolerance to leaf damage revealed significant nonlinear effects of damage on fitness (the proportion damage^2 term was positive and significant: F-value $= 77.58$, $P < 0.0001$). In addition, the nonlinear effects of damage on fitness were significantly different among maternal half-sibling families (the dam $\times$ proportion damage^2 term in the analysis of variance (ANOVA) was significant: F-value $= 2.59$, P $= 0.0044$); however, there was no evidence of nonlinear effects of damage on fitness among the paternal half-sibling families (the sire $\times$ proportion damage^2 term was not significant: F-value $= 1.29$, $P = 0.2312$).

The presence of nonlinear effects would mean that a linear function does not adequately describe tolerance given that some families might express higher levels of fitness at moderate levels of damage compared to other families. Thus, we performed a sequential polynomial regression analysis to determine the relative contributions of the linear and nonlinear components when estimating tolerance to leaf damage among maternal half-sibling families. We performed this analysis using all data as well as by each maternal half-sibling family. Untransformed relative fitness was the response variable in this analysis, with proportion leaf damage as the predictor variable in the linear regression, proportion damage^2 as the predictor variable in the quadratic regression, and proportion damage^3 in the cubic regression. SCORR1(SEQTESTS) was used in PROC REG (SAS version 9.1) to obtain squared sequential semipartial correlation coefficients and sequential tests of the predictor variables.

The sequential polynomial regression analysis revealed that the majority of the variance in fitness in response to varying levels of damage could be explained by the linear term in the regression ($r^2 = 0.33$). The quadratic term was significant, however, and added 11% explanatory power to the model. The cubic term added only approximately 1% more information in the regression, and was not statistically significant. The separate analyses on each maternal half-sibling family indicated that tolerance to leaf damage of some maternal lines could be best explained by the linear term in the regression, whereas in others the quadratic term added explanatory power to the model. Given that the quadratic term was not significant among all maternal families, and that the majority of the variance was explained by the linear term among all maternal families, we opted to use only the linear term when operationally estimating tolerance in the regression of fitness on the proportion of leaves damaged.

Costs of tolerance and resistance

Two types of costs were assessed: the potential for trade-offs in the form of a correlation between resistance and tolerance, and allocation, or fitness costs, of tolerance. Using paternal half-sibling families, we assessed the potential for a trade-off between resistance and tolerance, and the potential for fitness costs of both types of tolerance. Preliminary analyses showed only marginally significant genetic variation for tolerance to glyphosate among maternal half-sibling families ($P = 0.0769$). For this reason, trade-offs across maternal half-sibling families between tolerance to glyphosate and resistance were not determined, nor was the potential for a fitness cost of tolerance to glyphosate examined.

We tested for the presence of fitness costs by determining if a significant genetic covariance existed between family mean relative fitness, calculated from individuals in the absence of glyphosate, and family mean level of glyphosate tolerance. Using the same set of data to estimate both tolerance and the cost of tolerance to glyphosate produces an artifactual covariance between the two measures; following standard methods we subtracted this covariance from the calculated covariance for an unbiased estimate (Mauricio et al. 1997; Tiffin and Rausher 1999). This correction is not necessary for tolerance to leaf damage, as only the fitness of the sprayed individuals was used to determine this tolerance measure. Standard errors of the covariances between tolerance and fitness in the control plots were made by jackknifing sire half-sibling family line estimates (Gray and Schucany 1972) and a one-tailed t statistic was then used to calculate a P-value for the confidence interval to test that the covariances were less than zero.

To estimate the potential for a trade-off between tolerance and resistance, and a correlation between the two estimates of tolerance, genetic covariances between the traits were resampled by the jackknife procedure for either paternal or maternal half-sibling families. A one-tailed t statistic was then used to calculate a P-value for the confidence interval to determine if the genetic covariances between the two measures were less than zero, indicating a negative relationship between traits and thus the presence of a trade-off.

Selection on tolerance and resistance

The partial regression analysis described by Rausher (1992) was used to assess the pattern and magnitude of selection on tolerance and resistance in the presence of glyphosate. This analysis is based on genotypic family values rather than phenotypic values; as such the estimated selection gradients are unbiased by environmental covariances between traits and fitness (Rausher 1992; Mauricio and Mojonnier 1997; Stinchcombe et al. 2002). In addition, it is a relevant method for measuring selection on a trait such as tolerance, because tolerance cannot be measured on a single individual (Stinchcombe et al. 2002). Before conducting the analyses, tolerance and resistance values were standardized to a mean of zero and a standard deviation of one. For all analyses, the response variable was the residual of relative fitness after the effects of block were removed to minimize the effects of spatial variation.

Selection gradients were estimated for the traits that exhibited genetic variation. Using paternal half-sibling families, we

estimated selection on resistance and tolerance to leaf damage and tolerance to glyphosate ($W_d - W_u$), whereas selection on resistance and tolerance to leaf damage was assessed using maternal half-sibling families. Joint analyses of selection were performed by regressing fitness on resistance and the two tolerance measures using either paternal half-sibling or maternal half-sibling family means, following standard methods (Rausher 1992; Mauricio et al. 1997; Tiffin and Rausher 1999). A regression model that included only linear terms was used to estimate directional selection gradients for each of the three traits, whereas stabilizing/disruptive and bivariate nonlinear selection gradients (correlational selection) were estimated from the full model, including linear, quadratic, and interaction terms (Lande and Arnold 1983; Brodie et al. 1995).

A statistical artifact can obscure the relationship between fitness and tolerance when estimating selection on tolerance. If there is a strong positive correlation between the mean and the variance of fitness across half-sibling families, those families with low mean fitness are constrained by their low variance in fitness to have flatter slopes in the linear regressions of fitness on damage, and thus be scored as more tolerant (see Agrawal et al. 2004). Because these "highly tolerant" lines also exhibit low fitness, the selection analysis might aberrantly show a pattern of negative directional selection. The correlation between average fitness and variance in fitness of the maternal half-sibling families in our data was high ($r^2 = 0.87$), and remained high even after transformation ($r^2 = 0.84$). To address this, we performed a subsampling of the data in the selection analyses following Agrawal et al. (2004) to determine if the pattern of selection on tolerance either in the absence or presence of glyphosate was a genuine biological phenomenon or statistical artifact. We did this by repeating the selection analyses leaving out 25% and 50% of the families with the lowest variance in fitness, such that the families whose fitness variance was most likely to constrain their estimated slopes (tolerance) were removed. As the fitness costs analysis can also be affected by this potential statistical artifact, we subsampled the families when estimating the potential for costs of tolerance to glyphosate ($W_d - W_u$), and costs of tolerance to leaf damage in addition to the analyses of selection on tolerance. Further, using the same set of data to estimate both tolerance to glyphosate and selection on this trait can potentially produce a biased selection gradient much the same as the artifactual covariance that affects the fitness cost analysis. However, there is currently no viable method of removing this artifactual covariance in a joint analysis of selection.

Table 1. Mixed model analysis of variance for tolerance to glyphosate application. A significant interaction between Sire and/or Dam and Treatment indicates the presence of genetic variation for tolerance to glyphosate. The dependent variable, relative fitness, was square-root (y+1) transformed prior to analysis. For random effects we present the chi-square value from a likelihood-ratio test, and for fixed effects we present the *F*-statistic.

Source	F-statistic or χ^2	P-value
Diallel	$F_{1,1508}=93.71$	<0.0001
Treatment[1]	$F_{1,2}=46.48$	0.0207
Sire (Diall)	$F_{10,1508}=1.67$	0.0826
Dam (Diall)	$F_{10,1508}=2.98$	0.0010
Sire (Diall) × Dam (Diall)	$F_{37,1508}=0.78$	0.8249
Sire (Diall) × Treatment	$F_{10,1508}=2.12$	0.0203
Dam (Diall) × Treatment	$F_{10,1508}=1.69$	0.0769
Sire (Diall) × Dam (Diall) × Treatment	$F_{37,1508}=1.58$	0.0157
Block[1]	$\chi^2=0$	1.0000
Block × Treatment[1]	$\chi^2=28.1$	<0.0001

[1]Whole-plot factors; subplot factors are unmarked.

Results

EFFECT OF GLYPHOSATE ON FIELD INDIVIDUALS

Unsurprisingly, glyphosate application significantly decreased the fitness of sprayed individuals. Although fewer than 20% of the experimental plants died as a result of being sprayed (153 of 804), the fitness of sprayed plants was significantly lowered (F-value for the treatment effect = 46.48, $P = 0.0207$, Table 1) by approximately 80% compared to the control plants. Of the roughly 55% of sprayed individuals that were given a fitness score of "0," 294 individuals survived application by the herbicide yet did not produce seed. Thus, it appears glyphosate application affected plant reproduction more than plant survival. All sprayed plants sustained significant damage from glyphosate, with the proportion leaf damage ranging from 0.52 to 0.79 across both maternal and paternal half-sibling families.

GENETIC VARIATION FOR TOLERANCE

ANOVA revealed significant additive genetic variation among lines for both measures of tolerance. The sire by treatment interaction was significant in the analysis of tolerance to glyphosate (F-value = 2.12, $P = 0.0203$; Table 1) whereas both the sire by proportion damage and the dam by proportion damage terms were significant in the analysis of tolerance to leaf damage (F-value, sire interaction = 1.90, $P = 0.0425$, F-value, dam interaction = 3.78, $P < 0.0001$; Table 2). In addition, for both measures of tolerance, there was a significant dam × sire × treatment (or proportion damaged) interaction (Tables 1 and 2), indicating the presence of nonadditive genetic variation for tolerance.

GENETIC VARIATION FOR RESISTANCE

ANOVA indicated significant genetic variation for the proportion of the plant that was damaged following glyphosate application, meaning that its complement, resistance to glyphosate, also

Table 2. Analysis of covariance (ANCOVA) for tolerance to leaf damage following glyphosate application. A significant interaction between Sire and/or Dam and proportion damage indicates the presence of genetic variation for tolerance to leaf damage. The dependent variable, relative fitness, was square-root (y+1) transformed prior to analysis.

Source	df	Type III SS	*F*	*P*-value
Block	1	0.43	12.55	0.0004
Diallel	1	0.49	14.26	0.0002
Proportion damage	1	14.34	418.79	<0.0001
Sire (Diall)	10	0.93	2.71	0.0029
Dam (Diall)	10	2.03	5.92	<0.0001
Sire × Dam (Diall)	37	3.79	2.99	<0.0001
Proportion damage × Sire (Diall)	10	0.65	1.90	0.0425
Proportion damage × Dam (Diall)	10	1.30	3.78	<0.0001
Proportion damage × Sire × Dam (Diall)	37	2.88	2.27	<0.0001
Error	675	23.12		

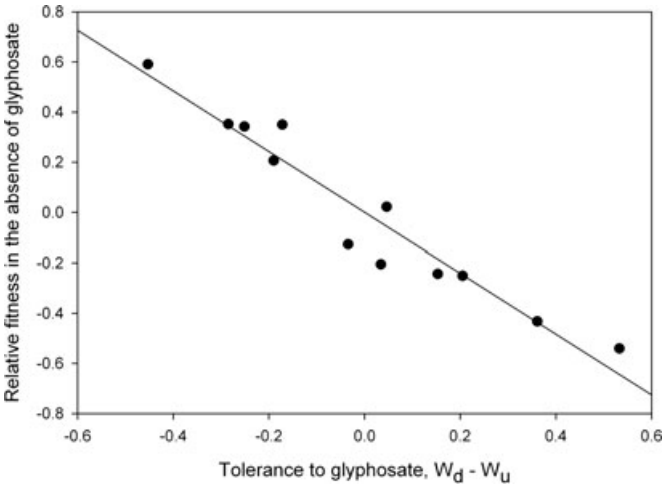

Figure 2. Relationship between fitness in the absence of glyphosate and tolerance to glyphosate among sire half-sibling families. Values of tolerance to glyphosate are based on fitness measured in both the presence and absence of glyphosate. The genetic covariance between fitness in the absence of glyphosate and tolerance was −0.346, $P < 0.02$, indicating a significant fitness cost of tolerance.

exhibited significant additive genetic variation (Table 3). Both the sire and dam effects were significant in this ANOVA, indicating that resistance varied among both maternal and paternal half-sibling families. Family mean resistance to leaf damage varied from 0.22 to 0.37 across paternal half-sibling families and from 0.20 to 0.40 across maternal half-sibling families, with overall means of both maternal and paternal families approximately equal to 0.28.

COSTS OF TOLERANCE: FITNESS COSTS AND CORRELATIONS

Tolerance to glyphosate ($W_d - W_u$) involves a significant fitness cost. The corrected covariance was −0.346 (jackknifed 95% CI = 0.23, $P < 0.02$). The coefficient of correlation between fitness in the absence of glyphosate and tolerance was −0.964 (jackknifed 95% CI = 0.004, $P < 0.0001$; Fig. 2). There was no evidence for nonlinear costs of tolerance to glyphosate (the tolerance^2 term from a regression of fitness in the absence of glyphosate on tolerance was not significant $F = 0.01$, df = 1, 11, $P = 0.9329$; sensu Tiffin and Rausher 1999). The fitness cost of tolerance to glyphosate was not estimated for maternal half-sibling families, because maternal lines were only approaching statistical significance for genetic variation for tolerance (Table 1, $P = 0.0769$).

Table 3. Analysis of variance for resistance to leaf damage. The dependent variable, proportion of leaf damage, was arcsine square-root transformed before analysis.

Source	df	Type III SS	*F*	*P*-value
Block	1	5.49	30.35	<0.0001
Diallel	1	2.68	14.81	0.0001
Sire (Diall)	10	3.62	2.00	0.0304
Dam (Diall)	10	3.88	2.15	0.0193
Sire × Dam (Diall)	37	4.00	0.60	0.9730
Error	735	132.95		

We performed the analysis of fitness costs of glyphosate tolerance after removing families with the lowest 25% and 50% fitness variance to correct for a potential statistical artifact that would bias the result of a fitness cost. We found no evidence, however, that the "high tolerance/low fitness" lines were biasing the fitness costs analysis: the corrected covariance using 75% of the paternal half-sibling families with the highest fitness variance ($N = 9$) was -0.313 ± 0.23, and the corrected covariance with 50% of the paternal half-sibling families with the highest fitness variance ($N = 6$) was -0.34 ± 0.30. Neither of the confidence intervals at either sampling depth overlapped zero, providing strong evidence that the mean/variance correlation in fitness in our data did not bias our finding of a fitness cost of glyphosate tolerance.

Both the maternal and paternal half-sibling families exhibited a significant fitness cost of tolerance to leaf damage (covariance ± 95% CI paternal half-sibling families = -0.157 ± 0.023, correlation ± 95% CI = -0.437 ± 0.05; maternal half-sibling families = -0.189 ± 0.014, correlation ± 95% CI = -0.548 ± 0.04). These covariances were significantly different from zero after subsampling the data to correct for the mean/variance correlation

Table 4. Genetic covariance matrix between tolerance to glyphosate ($W_d - W_u$), tolerance to leaf damage (as determined by regressions of fitness on leaf damage), and resistance to leaf damage (1−proportion of total leaves exhibiting damage). All trait values were standardized to a mean of "0" and a standard deviation of "1." Values of the genetic covariance between traits are followed by their jackknifed 95% CI. Sire half-sibling family values are on the upper diagonal and dam half-sibling family values are on the lower diagonal. Tolerance to glyphosate ($W_d - W_u$) was not estimated among dam half-sibling families, as there was only marginally significant evidence of genetic variation for this trait (Table 1). All covariances were significantly different from zero at $P \leq 0.0001$.

Trait	Resistance to leaf damage	Tolerance to leaf damage	Tolerance to glyphosate
Resistance to leaf damage	1.0	−0.159 (−0.11, −0.21)	−0.406 (−0.36, −0.45)
Tolerance to leaf damage	−0.529 (−0.48, −0.57)	1.0	0.264 (0.20, 0.33)
Tolerance to glyphosate			1.0

(covariance ± 95% CI between fitness and tolerance using 75% of the families, paternal half-sibling families = −0.152 ± 0.030, maternal half-sibling families = −0.195 ± 0.013). Thus it appears that there are significant fitness costs associated with both tolerance to glyphosate and tolerance to leaf damage following glyphosate application. There was no evidence of a nonlinear fitness cost associated with tolerance to leaf damage among either the maternal or paternal half-sibling families (the tolerance to leaf damage^2 term from a regression of fitness in the absence of glyphosate on tolerance was not significant: maternal families $F = 0.24$, df = 1, 11, $P = 0.6366$, paternal families $F = 0.25$, df = 1, 11, $P = 0.6298$; sensu Tiffin and Rausher 1999).

Another possible constraint on the evolution of tolerance is a negative correlation with resistance (Fineblum and Rausher 1995). Both measures of tolerance were significantly negatively genetically correlated to resistance. The genetic covariance between resistance and tolerance to leaf damage was −0.529 (Table 4) among maternal half-sibling families, and −0.159 (Table 4) among paternal half-sibling families. There was also evidence of a trade-off between tolerance to glyphosate and resistance to leaf damage among paternal half-sibling families, with the covariance between the two traits equal to − 0.406 (Table 4). The two measures of tolerance were positively genetically correlated, with a covariance of 0.264 (Table 4). It is important to acknowledge that we included individuals from different populations in our crossing design such that population of origin was not controlled. This means that the correlations uncovered in our analyses could be due to either linkage disequilibrium or to pleiotropy (Falconer and Mackay 1996).

SELECTION ON TOLERANCE AND RESISTANCE

The multiple family-mean linear regression estimating the joint pattern of selection on resistance and tolerance to leaf damage in the presence of glyphosate, as well as tolerance to glyphosate ($W_d - W_u$), uncovered evidence of positive directional selection on resistance to leaf damage (Fig. 3A) and negative directional selection on tolerance to leaf damage (Fig. 3B) among both paternal and maternal half-sibling families (Tables 5 and 6). There was no evidence that tolerance to glyphosate ($W_d - W_u$), was under either positive or negative directional selection among paternal half-sibling families (Table 6) in the presence of glyphosate. This suggests that the costs of tolerance to glyphosate, as reported

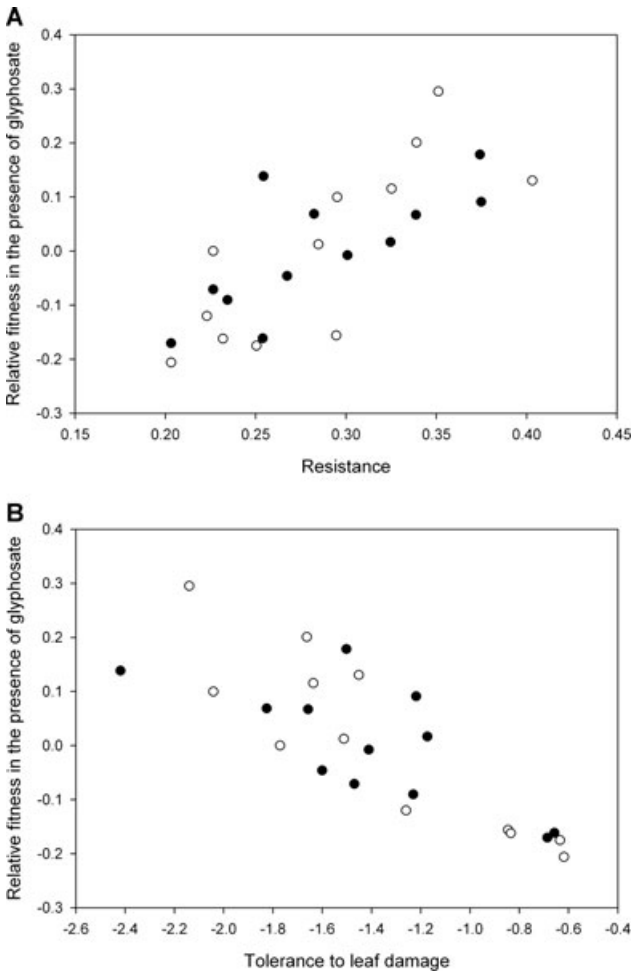

Figure 3. Relationship between relative fitness in the presence of glyphosate and (A) resistance to glyphosate and (B) tolerance to leaf damage following glyphosate application. Sire half-sibling families are represented by • whereas dam half-sibling families are marked by ∘. The linear selection gradients for resistance and tolerance to leaf damage are presented in Table 5 (maternal half-sibling families) and Table 6 (paternal half-sibling families).

Table 5. Regression analyses showing the linear, quadratic, and correlational selection gradients acting on tolerance to glyphosate, tolerance to leaf damage following glyphosate application, and resistance to leaf damage among maternal half-sibling families. Maternal half-sibling family values were standardized to a mean of "0" and a standard deviation of "1" before analyses. Linear values are from a regression with no quadratic or interaction terms included.

Trait	df	Estimate	Standard error	*P*-value
Resistance to leaf damage	1	0.075	0.017	0.0015
Tolerance to leaf damage	1	−0.109	0.017	0.0001
Resistance to leaf damage × tolerance to leaf damage	1	−0.060	0.025	0.0517
Resistance to leaf damage^2	1	−0.012	0.014	0.6552
Tolerance to leaf damage^2	1	−0.040	0.021	0.3805

above, outweighed its benefits in this particular field season. In a regression including the quadratic terms, there was no evidence of either stabilizing or disruptive selection on either resistance or tolerance among sire and dam families. However, among maternal half-sibling families, there was a marginally significant negative two-way interaction between resistance and tolerance to leaf damage (Table 5) reflecting negative correlational selection on the two traits. There was no evidence of negative correlational selection on resistance and either measure of tolerance among the sire half-sibling families (Table 6).

Table 6. Regression analyses showing the linear, quadratic, and correlational selection gradients acting on tolerance to glyphosate, tolerance to leaf damage following glyphosate application, and resistance to leaf damage among paternal half-sibling families. Paternal half-sibling family values were standardized to a mean of "0" and a standard deviation of "1" before analyses. Linear values are from a regression with no quadratic or interaction terms included.

Trait	df	Estimate	Standard error	*P*-value
Resistance to leaf damage	1	0.067	0.011	0.0002
Tolerance to leaf damage	1	−0.067	0.010	0.0002
Tolerance to glyphosate	1	−0.015	0.011	0.1977
Resistance to leaf damage × tolerance to leaf damage	1	0.009	0.026	0.7442
Resistance to leaf damage × tolerance to glyphosate	1	0.030	0.054	0.6311
Tolerance to leaf damage × tolerance to glyphosate	1	0.004	0.070	0.9549
Resistance to leaf damage^2	1	0.076	0.030	0.3357
Tolerance to leaf damage^2	1	0.002	0.031	0.9666
Tolerance to glyphosate^2	1	−0.026	0.034	0.7398

The selection analysis on tolerance, like the fitness costs analysis, can be biased by the "high tolerance/low fitness" artefact that can arise from estimating tolerance. We addressed this possibility in the analyses of selection on both tolerance to leaf damage and tolerance to glyphosate in the presence of glyphosate. We again removed families with the lowest 25% and 50% fitness variance and performed the selection analysis. We found no evidence that families with low variance in fitness were biasing the analyses of selection on tolerance to leaf damage. When removing 25% of the families with the lowest fitness variance ($N = 9$ in the analysis) and 50% of the families with the lowest fitness variance ($N = 6$ in the analysis) the pattern of negative directional selection remained significant (maternal half-sibling families: using 75% of the families $\beta = -0.127$, $P = 0.0013$; 50% of the families $\beta = -0.152$, $P = 0.0008$, paternal half-sibling families: using 75% of the families $\beta = -0.066$, $P = 0.0037$; 50% of the families $\beta = -0.110$, $P = 0.0490$). Thus we provide strong evidence that the pattern of selection on tolerance to leaf damage in the presence of glyphosate is one of negative directional selection. There was no evidence of selection on tolerance to glyphosate in the presence of glyphosate among sires in this field season; performing the subset sampling correction did not provide evidence that the mean/variance correlation affected our results (bottom 25% families removed $\beta = -0.009$, $P = 0.6281$; bottom 50% families removed $\beta = 0.099$, $P = 0.4149$).

Discussion

CONSTRAINTS IN THE FORM OF CORRELATIONS BETWEEN TOLERANCE AND RESISTANCE

Theoretical demonstrations of the joint evolution of resistance and tolerance generally predict that resistance and tolerance should show a pattern of mutual exclusivity (Fineblum and Rausher 1995; Mauricio et al. 1997; Abrahamson and Weis 1997). These predictions are based on the assumption that complete resistance and complete tolerance are redundant and incur fitness costs, and that having both defensive strategies leads to a cost greater than would be experienced if only one strategy was employed. Although a few empirical studies have found negative genetic correlations between resistance and tolerance (Fineblum and Rausher 1995; Stowe 1998; Fornoni et al. 2003) the majority have not (Simms and Triplett 1994; Mauricio et al. 1997; Tiffin and Rausher 1999; Stinchcombe and Rausher 2002; Weinig et al. 2003; Carr et al. 2006).

We find such a cost in this study. This result is similar to the work of Fineblum and Rausher (1995) who found a trade-off between resistance and tolerance to herbivory in *I. purpurea*. In comparison, Tiffin and Rausher (1999) and Simms and Triplett (1994), also using *I. purpurea*, found no such correlations. One potential explanation for the variance in results is that the experimental

plants used in each study were derived from different source populations with different evolutionary histories. Another explanation is that the level of damage the plants experienced in this study was higher than the level of damage from herbivores (Tiffin and Rausher 1999), or pathogens (Simms and Triplett 1994), such that the strength and novelty of selection by glyphosate potentially affects the expression of the correlations. Further, the agents of selection between experiments were very different, and virtually nothing is known about the mechanisms underlying resistance and/or tolerance to glyphosate in *I. purpurea*. The presence of negative genetic correlations between resistance and tolerance in our study is, however, evidence of a trade-off between the traits, and provides support for a mechanism by which the evolution of defense can be constrained.

THE SELECTIVE LANDSCAPE OF CONSTRAINTS ON TOLERANCE

The linear selection analyses on glyphosate defense traits indicated strong negative selection against tolerance to leaf damage, strong positive selection for resistance to leaf damage, and no evidence of selection on tolerance to glyphosate in the presence of the herbicide. It was our expectation that tolerance to leaf damage would exhibit a pattern of positive selection; the apparent negative selection was likely caused by the costs of this measure of tolerance outweighing any potential benefit. The finding of negative selection on tolerance to leaf damage yet positive selection on resistance suggests that continued selection by glyphosate should act to both decrease the level of tolerance to leaf damage in the population and increase the level of resistance over time.

Counter to our expectations, the joint selection analysis of the paternal families showed no evidence for positive selection on tolerance to glyphosate ($W_d - W_u$). However, in the absence of glyphosate, a highly significant negative correlation existed between this measure of tolerance and fitness such that this study population was exhibiting a fitness cost of tolerance to glyphosate. This is in agreement with previous work finding a fitness cost of tolerance to glyphosate in *I. purpurea* (Baucom and Mauricio 2004), and provides further verification of a mechanism by which tolerance can be maintained at an intermediate level in the study population, if there is a benefit of being tolerant in some years, as has been previously seen (Baucom and Mauricio 2004). We did not find a nonlinear cost of tolerance in the absence of glyphosate, nor did we find evidence of stabilizing selection on tolerance in the presence of glyphosate. Taken together, these findings suggest that, although there is evidence of genetic variation for tolerance to glyphosate, the level of tolerance is not maintained in this system by stabilizing selection as mediated by the costs and benefits of tolerance. In populations of morning glories, the level of tolerance to glyphosate is likely governed by temporally fluctuating selection such that crop rotations potentially act to maintain tolerance at intermediate levels. This assertion is dependent on the presence in some years of a benefit of tolerance to the application of glyphosate.

The quadratic selection analyses uncovered evidence of a marginally significant negative interaction between resistance and tolerance to leaf damage among maternal half-sibling families, indicating that correlational selection is promoting either resistance or tolerance to leaf damage, but not both. However, this result was not present among the paternal half-sibling families. Finding this effect among the maternal half-sibling families but not the paternal families suggests the possibility that nonadditive genetic effects underlie this result. This is because the maternal contribution to the variance of a trait includes a nonadditive portion through the maternal inheritance of the chloroplast genome and other plastids. If tolerance or resistance were governed, at least in part, by a gene on the chloroplast genome, selection on the traits might be expected to be more intense through the maternally inherited component. This assertion is highly speculative, but is a possibility, given that glyphosate interacts with the shikimate acid pathway of plants in a reaction that occurs in the cytosol of the chloroplast (Della-Cioppa et al. 1986). Alternatively, the lack of correlative selection among the paternal half-sibling families could be due to limited statistical power in a multiple regression with nine independent variables and only 12 observations.

Although we did not uncover evidence of direct selection on tolerance to glyphosate in this study, we did find evidence that its evolutionary trajectory is influenced through indirect selection via the correlations between traits. This finding represents another type of constraint on the evolution of increased tolerance and, as well, a potential constraint on the evolution of resistance. Tolerance to glyphosate was negatively correlated to resistance, and positively correlated to tolerance to leaf damage. Thus, tolerance to glyphosate is under indirect negative selection through these correlations—positive direct selection for increased resistance in the population will lead to negative indirect selection on tolerance to glyphosate, whereas the negative direct selection on tolerance to leaf damage will lead to negative indirect selection on tolerance to glyphosate. Likewise, if selection on tolerance to glyphosate were positive, as has been found in previous years, the negative correlation between the traits could act to constrain an increase in the mean value of resistance, thus constraining its evolution.

Conclusions

The presence of genetic variation in both resistance and tolerance to glyphosate in *I. purpurea* suggests two different types of defensive adaptations are possible given herbicide application, rather than just a single defensive strategy. Furthermore, the

negative correlations between resistance and tolerance to glyphosate provide evidence that the evolutionary trajectory of one trait could constrain the trajectory of the other. The presence of negative correlational selection on resistance and tolerance to leaf damage suggests that the two traits should evolve to be mutually exclusive adaptations given continued selective pressure by glyphosate application. That this result was uncovered only among maternal half-sibling families and not among the paternal half-sibling families suggests this finding, as well as the inheritance of glyphosate defense traits, warrants further study. It is important to note that the lines used in this field experiment were chosen based on their vegetative response to glyphosate rather than based on their fitness response, and that this initial family selection scheme could be influencing our results. However, the negative relationship between fitness and tolerance in the absence of glyphosate provides further verification that fitness costs are associated with tolerance. This result is in agreement with a previous field experiment in which selfed seed from field-collected maternal lines were used rather than lines chosen for a specific reason as in this experiment. These two findings, that of a fitness cost of tolerance, and costs of tolerance in the form of negative genetic correlations between tolerance and resistance, provide empirical support for the predictions of the cost/benefit models of the evolution of defensive traits.

Selection by glyphosate has become a predominant force in the agro-ecosystem (Baucom and Mauricio 2004), and will continue to be so as long as agriculturists rely heavily on this single herbicide. The widespread adoption of Roundup Ready crops such as soybean, cotton, maize and canola will likely continue this Roundup reliance for quite some time. This system thus represents an expansive experiment in evolutionary biology, and one that merits continued study.

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