

OVEREXPRESSION OF A NATIVE GENE ENCODING 5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE (EPSPS) MAY ENHANCE FECUNDITY IN *ARABIDOPSIS THALIANA* IN THE ABSENCE OF GLYPHOSATE

Zachery T. Beres,^{1,*} Xiao Yang,^{2,*} Lin Jin,[†] Wanying Zhao,[†] David M. Mackey,^{†,‡} and Allison A. Snow^{*}

^{*}Department of Evolution, Ecology, and Organismal Biology, Ohio State University, Columbus, Ohio 43210, USA; [†]Department of Horticulture and Crop Science, Ohio State University, Columbus, Ohio 43210, USA; and [‡]Department of Molecular Genetics, Ohio State University, Columbus, Ohio 43210, USA

Editor: Hafiz Maherali

Premise of research. Strong environmental selection pressures can lead to rapid adaptation and the opportunity to study evolutionary dynamics in real time. A prime example is the recent evolution of resistance to the herbicide glyphosate, the active ingredient in Roundup, in more than 35 weed species. Mechanisms for glyphosate resistance include gene amplification and overproduction of its target enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), but little is known about whether these genetic changes are associated with differing fitness in glyphosate-free environments. Such fitness effects could have major implications for anticipated changes in the frequency of resistance traits without continued exposure to the selective pressure.

Methodology. We used transgenic *Arabidopsis thaliana* as a model system to test for the effects of overproducing EPSPS on plant growth and reproduction. In a previous study, we developed six independent transgenic lines that overexpress a native *EPSPS* gene driven by the CaMV35S promoter (designated OX) and seven independent empty vector lines (designated EV). Here, we compared phenotypic traits among these lines and their wild-type parents in greenhouse experiments.

Pivotal results. Two of the OX lines produced 23%–37% more seeds per plant than the wild-type line, respectively, and none showed evidence of a fitness penalty. In contrast, the performance of the EV lines was similar to, or somewhat worse than, that of the wild-type line. Despite considerable variation among lines, the OX lines had greater fecundity than the wild-type or EV lines overall.

Conclusions. Our results suggest that overproduction of EPSPS in *Arabidopsis* does not have a fitness cost and might confer a fitness benefit under the examined growth conditions. Further basic research on how surplus EPSPS affects plant growth is warranted. We hypothesize that similar effects could occur in weed species that overproduce EPSPS, but the few studies that address this question have shown mixed results and no evidence for a fitness benefit to date.

Keywords: fitness effects, glyphosate-resistant, pesticide, transgenic, weeds.

Introduction

The pervasive evolution of pesticide resistance is a classic example of rapid adaptation, with major implications for agriculture and public health (Neve et al. 2008; REX Consortium 2013). Extremely strong selection pressure from herbicides, insecticides, and fungicides has resulted in a wide range of resistance mechanisms across taxa, sometimes involving similar types of adaptations (Délye et al. 2013 and references

therein). For example, target-site mutations that increase the production of the target protein have been documented in fungi (Leroux and Walker 2011), insects (Karasov et al. 2010), and agricultural weeds (Délye et al. 2013; Heap 2017; Laforest et al. 2017; Tranel 2017). Aphids (*Myzus perscae*) and *Culex* mosquitoes have evolved resistance to organophosphate insecticides by overproducing esterase (Field and Devonshire 1997; Paton et al. 2000). Weedy plant species have evolved resistance to the herbicide glyphosate by several mechanisms, including overproduction of glyphosate's target enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC2.5.1.19), as well as target-site codon substitutions, and/or nontarget-site adaptations such as reduced translocation (Sammons and Gaines 2014).

Glyphosate (the active ingredient in Roundup) is a systemic, broad-spectrum herbicide used widely in row crops, orchards, and vineyards (Duke and Powles 2008). The evolution of

¹ Author for correspondence; e-mail: beres.36@osu.edu.

² Current address: Ministry of Education Key Laboratory for Biodiversity and Ecological Engineering, Institute of Biodiversity Science, Fudan University, Songhu Road 2005, Shanghai 200438, China.

Manuscript received October 2017; revised manuscript received December 2017; electronically published March 26, 2018.

glyphosate-resistant weeds has been facilitated by overreliance on this popular once-in-a-century herbicide, especially in conjunction with glyphosate-resistant crops (Duke and Powles 2008). Currently, more than 35 weed species have evolved resistance to glyphosate (Heap 2017). In surveys of farmers in the United States, glyphosate-resistant weeds such as *Amaranthus palmeri* have been listed in the top tier of the most damaging weeds (Heap 2014). Thus, glyphosate resistance has become a major economic and environmental problem in the United States and elsewhere (Mortensen et al. 2012; Shaner et al. 2012).

Efforts to delay or manage pesticide resistance in agroecosystems require a thorough understanding of resistance mechanisms, their fitness effects, and the organism's life history, demography, ecology, mating system, and population genetics (Roush and Tabashnik 1990; Jasieniuk et al. 1996). As studies of the genetic basis of resistance become more sophisticated, complementary research on underlying fitness effects is needed to help predict the persistence and spread of resistant genotypes (Kliot and Ghanim 2012; REX Consortium 2013; Steinbach et al. 2017). Here, we use the term “underlying fitness effects,” or, simply, “fitness effects,” to refer to the net fitness costs or benefits for the organism that occur without exposure to a particular pesticide. Fitness costs can result from various mechanisms, including pleiotropic effects of resistance and reallocation of resources needed for growth and reproduction (Délye et al. 2013). Although it is widely assumed that pesticide resistance is associated with fitness costs, such effects may range from severely deleterious to neutral (Kliot and Ghanim 2012; Délye et al. 2013) or, more rarely, beneficial (Wang et al. 2010). A genetic resistance mechanism that does not confer a fitness cost is expected to exacerbate challenges for reducing its frequency and proliferation within and among pest populations (Délye et al. 2013; Wu et al. 2017).

Further research on the fitness effects of herbicide resistance is warranted because many studies, to date, are inconclusive or incomplete (Vila-Aiub et al. 2011; Giacomini et al. 2014).

Designing studies that control for genetic background can be challenging, and results based on a small number of susceptible versus resistant biotypes from different populations lack the appropriate controls (Vila-Aiub et al. 2009, 2011; Giacomini et al. 2014). Another challenge is that researchers typically focus on components of fitness rather than the entire plant life cycle, and susceptible versus resistant genotypes often are compared for only a single generation under a limited range of environmental conditions. Therefore, these studies might not reflect patterns that would be seen under multigenerational field conditions. Furthermore, newly evolved fitness costs may diminish over time due to selection for compensatory responses (Vila-Aiub et al. 2011; Darmency et al. 2015). Thus, a combination of multiple studies with different approaches is needed to test for the fitness effects of herbicide resistance.

To date, at least 10 weed species have acquired glyphosate resistance by overproducing EPSPS, a key enzyme in the shikimate pathway (table 1). The shikimate pathway is found in all plants and accounts for ~30% of a plant's carbon budget by providing substrate for the production of aromatic amino acids, auxin, lignin, and plant-defensive compounds (e.g., inducible antifungal agents, glucosinolates, alkaloids, and tannins; Herrmann and Weaver 1999; Tzin and Galili 2010; Maeda and Dudavera 2012). EPSPS plays a central role in plant growth and survival, and overproduction of this enzyme could incur a fitness cost (see “Discussion”), for example, under conditions of limited resources for growth. However, based on studies by Wang et al. (2014) and Yang et al. (2017b), we hypothesized that transgenic overproduction of EPSPS would be neutral or beneficial in terms of fitness-related traits (e.g., number of seeds produced) in glyphosate-free environments. For example, more abundant EPSPS might enhance the flux of carbon through the shikimate pathway and provide reserves of aromatic amino acids for downstream metabolic products (Pline-Srvic 2006). Any minor metabolic cost of overproducing EPSPS might be offset by benefits at various stages of the plant's life cycle.

Table 1
Examples of Weed Species That Overproduce EPSPS as a Mechanism for Glyphosate Resistance

Species	EPSPS copy numbers	Locations	References
<i>Amaranthus palmeri</i>	5–>100	Georgia	Gaines et al. 2010, 2011, 2013
<i>Amaranthus palmeri</i>	2–10	New Mexico	Mohseni-Moghadam et al. 2013
<i>Amaranthus spinosus</i> ^a	33–37	Mississippi	Nandula et al. 2013
<i>Amaranthus tuberculatus</i>	2–8	Illinois, Missouri	Lorenz et al. 2014
<i>Amaranthus tuberculatus</i>	2–10	Illinois, Kansas, Missouri, Nebraska	Chatham et al. 2015a
<i>Amaranthus tuberculatus</i>	3–10	Illinois	Chatham et al. 2015b
<i>Bromus diandrus</i>	10–36	South Australia	Malone et al. 2016
<i>Chloris truncata</i>	32–48	New South Wales, Australia	Ngo et al. 2017
<i>Conyza canadensis</i>	NR	Arkansas, Delaware, Ohio, Virginia	Dinelli et al. 2006
<i>Conyza canadensis</i>	NR	Lakonia, Greece	Tani et al. 2015
<i>Eleusine indica</i>	27–35	Southwest China	Chen et al. 2017
<i>Eleusine indica</i>	NR	Veracruz, Mexico	Gherekhloo et al. 2017
<i>Kochia scoparia</i>	9–16	Kansas	Jugalam et al. 2014
<i>Kochia scoparia</i>	3–10	Colorado, Kansas, North Dakota, South Dakota	Wiersma et al. 2015
<i>Kochia scoparia</i>	3–13	Colorado, Wyoming, Nebraska	Gaines et al. 2016
<i>Lolium perenne</i>	11–151	Arkansas	Salas et al. 2015
<i>Lolium rigida</i>	NR	North Victoria, Australia	Baerson et al. 2002

Note. The range of approximate copy numbers for amplification of the *EPSPS* gene is shown when reported. NR = not reported.

^a Likely acquired by hybridizing with *A. palmeri*.

To test our hypothesis, we used transgenic *Arabidopsis thaliana*, a small self-pollinating winter annual and model species for plant biology, to determine the effects of constitutively overproducing EPSPS on plant growth and reproduction under greenhouse conditions. Because we studied transgenic *Arabidopsis* rather than a field-collected glyphosate-resistant weed species, we were able to avoid linkage between resistance genes and their flanking regions, such as genes found within each extra copy of the very large EPSPS amplicon in *A. palmeri* (Molin et al. 2017). Furthermore, many weed species have field-evolved resistance to multiple herbicides, as well as multiple resistance mechanisms for the same herbicide (Délye et al. 2013; Korht et al. 2017), which complicates efforts to focus on each mechanism individually. Nearly all previous studies of transgenic plants that overproduce EPSPS have focused specifically on documenting glyphosate resistance, without considering the pleiotropic effects that this mechanism may have on plant fitness (Klee et al. 1987; Jones et al. 1996; Pline-Srvic 2006; but see Wang et al. 2014 and Yang et al. 2017b).

For the current study, we compared transgenic *Arabidopsis* lines with a native *EPSPS* gene driven by the CaMV35S promoter (denoted OX, for overexpression) with transgenic lines derived from the empty vector (denoted EV), which carried a nonfunctional gene. As presented in Yang et al. (2017a), the OX lines had greater *EPSPS* gene expression and therefore were more resistant to glyphosate than the EV lines or the wild-type parental line (fig. 1B). Here, we compared the fitness-related traits of plants from six independent OX lines, seven independent EV lines, and the wild-type parent in two greenhouse experiments, A and B. Our primary goal was to compare the total number of seeds produced per plant among lines as a proxy for fitness (Vila-Aiub et al. 2011). We also recorded the number of days to seedling emergence, the plant size prior to bolting, and the number of days to the onset of flowering, because these life-history traits also could be associated with fitness differences among genotypes (Campbell and Snow 2007). Although our experiments were conducted in the greenhouse rather than in the field, where environmen-

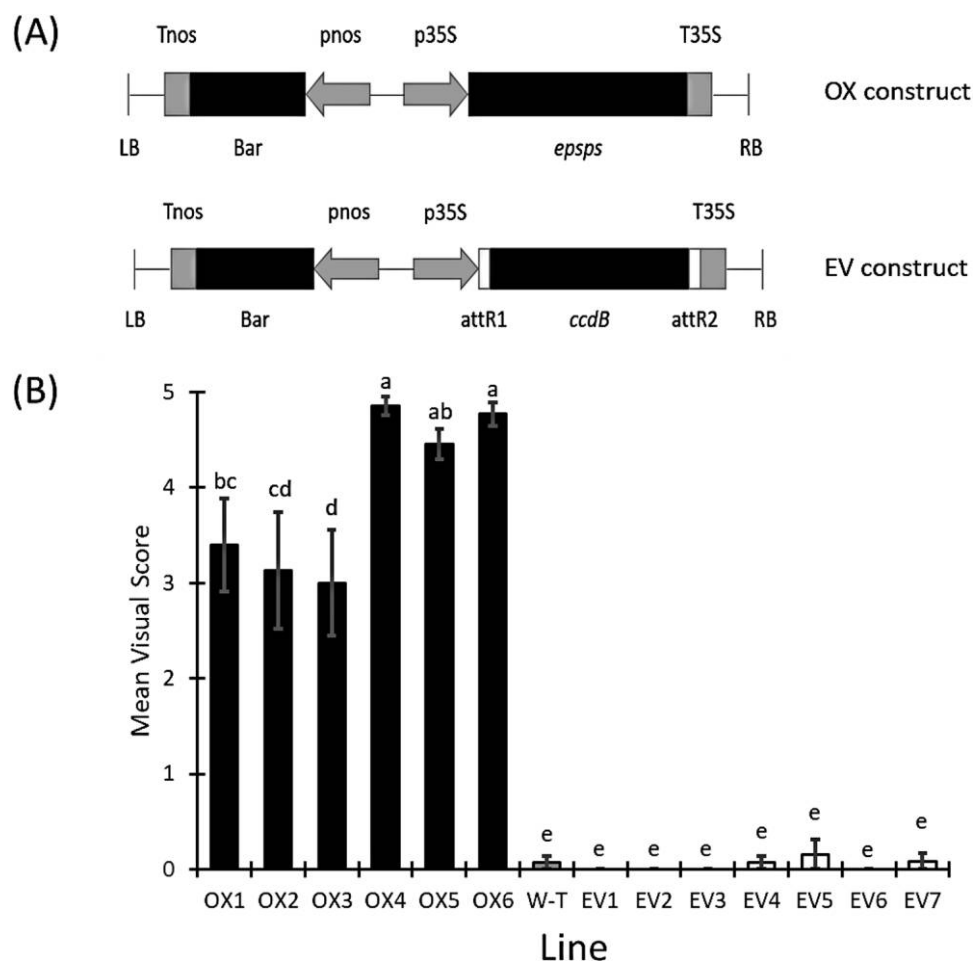


Fig. 1 A, Diagrams of the OX (*EPSPS* overexpression) and EV (empty vector, pB2WG7) constructs (from Yang et al. 2017a). In the OX construct, the entry clone-*EPSPS* complementary DNA sequence was inserted between the attR1 and attR2 sites. B, Relative glyphosate resistance of OX, EV, and wild-type *Arabidopsis* lines, showing means \pm 1 SE for visual damage 21 d after spraying with \times 0.5 glyphosate (from Yang et al. 2017a). Visual damage scores are based on a scale of 0–5; 0 indicates plants that died, and 5 indicates plants that were mostly green and developing new leaves. Means that do not share superscripts are significantly different at $P < 0.05$ (Tukey tests). $N = 11$ –15 plants per line. These figures were modified from Yang et al. (2017a).

tal conditions would be much more variable, this study serves as a starting point for understanding whole-plant responses to overproduction of *EPSPS* in *Arabidopsis*.

Material and Methods

Parent Material

Arabidopsis thaliana (thale cress; Brassicaceae) is a small Eurasian weed found in disturbed habitats and now occurs on several continents (Platt et al. 2010). The small genome, short generation time, and high levels of self-pollination in *Arabidopsis* have contributed to its widespread use as a model system in plant biology, ecology, and evolution (Mitchell-Olds 2001; Platt et al. 2010). We used the Columbia ecotype (Col-0) of *A. thaliana*, obtained from the Arabidopsis Biological Resource Center at Ohio State University (<https://abrc.osu.edu/>), as the parent material for our experimental lines. Col-0 is one of the most commonly used ecotypes in transgenic studies of *Arabidopsis*.

Development of Overexpressing and Empty Vector Transgenic Lines

Research with multiple independent lines for each transgene provides a way to minimize genetic background effects on traits of interest (Bergelson et al. 1996; Jackson et al. 2004). The development of lines for this study is described by Yang et al. (2017a) and summarized in figure 1A. Briefly, we developed *A. thaliana* lines with independent insertions of OX versus EV constructs. *Arabidopsis* has two native *EPSPS* loci (AT1G48860 and AT2G45300) that are highly expressed throughout development (Gaines et al. 2010; <http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). We chose to insert an extra copy of AT1G48860, due to its purported greater resistance to glyphosate than AT2G45300 in *Arabidopsis* (Lee 2006). To generate the *EPSPS* construct, the entry clone was introduced downstream of the CaMV35S promoter in the binary expression vector pB2WG7, which also carried the *Bar* gene for resistance to the herbicide glufosinate downstream from the nopaline synthase promoter (pnos, fig. 1A). The EV construct carried the *ccdB* gene from *Escherichia coli* instead of the *EPSPS* gene (fig. 1A). The *ccdB* gene has no known function in plants and is commonly used to create EVs. The OX (CaMV35S::*EPSPS*) construct was confirmed by sequencing, and both the OX and EV constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, which was used to transform *A. thaliana* (Col-0) by the floral dip method (Clough and Bent 1998).

Transgenic progeny were selected by spraying soil-grown seedlings on three consecutive days with 0.04% Basta (w/v; glufosinate) supplemented with 0.005% SilwetL-77 solution. Independent T1 lines with presumed single-insertion loci were identified by 3:1 segregation of glufosinate resistance in the T2 generation. These T2 lines were allowed to self, and homozygous T3 lines (those that segregated 4:0) were used in subsequent experiments. Using this procedure, we generated nine independent OX lines and nine independent EV lines.

Several lines were not used in the current study because two of the original nine OX lines did not exhibit greater resistance to glyphosate than the wild-type or EV controls and one of the

resistant OX lines had abnormal development and branching (Yang et al. 2017a). Therefore, three of the original nine OX lines could not be used. In addition, two of the nine EV lines were not used here because such replication of the EV control was deemed unnecessary. For ease of presentation, we labeled the OX lines 1–6 and the EV lines 1–7 based on the fecundity levels found in experiment B, with 1 having the most seeds per plant (same labels as those in Yang et al. 2017a). All six OX lines were more resistant to glyphosate than the wild-type and the seven EV lines (fig. 1B). Some of the OX lines were more resistant to glyphosate than others, and those that were most resistant, OX4–6, had greater *EPSPS* transcript levels than OX1–3 (Yang et al. 2017a). Resistance levels were assessed using visual scores from 0 (dead) to 5 (healthy, <10% visual damage; for further details, see fig. 1B and Yang et al. 2017a).

Experiments to Compare Growth, Flowering Time, and Fecundity

To test for phenotypic effects of overproducing *EPSPS* in the absence of glyphosate, we carried out two greenhouse experiments, A and B, in September 2015 and November 2015, respectively. The purpose of conducting two experiments was to scale up in the second one with more OX and EV lines and to test for consistent results for lines used in both experiments. Experiment A included four OX lines, four EV lines, and the wild-type Columbia line. For experiment B, we used all available OX lines (OX1–6), EV lines (EV1–7), and the wild-type line. Sample sizes were 45 plants per line in experiment A and 75 plants per line in experiment B, for a total of 1455 plants. The EV lines served as transgenic controls for the OX lines because they included the same selectable marker gene (*Bar*, for resistance to glufosinate) and the same promoters (fig. 1A).

Seeds were germinated in 8.5-cm-wide square pots filled with moistened Fafard no. 2 soil and thinned 1 wk after planting to one seedling per pot. Pots were randomly grouped in trays (analyzed as blocks), with two plants per line in each tray. Experiment B utilized three cohorts that were planted on consecutive days in a factorial design, such that there were multiple blocks per cohort. All trays were subirrigated as needed and rotated weekly to account for environmental variation. For both experiments, the greenhouse was maintained at 18°–21°C/23°–26°C (night/day), and supplemental lights (400-W metal halide) were used for 14 h d⁻¹. In experiment A, the plants were fertilized at 4 wk after planting with 50 mL of nutrient solution (180 ppm 20:10:20; <http://www.jpeters.com>). In contrast, in experiment B the plants were fertilized with 25 mL of the same nutrient solution at 3, 4, and 5 wk after planting to provide a greater and more even level of nutrient availability. As shown below, the plants in experiment B grew larger and flowered later than those in experiment A.

During the first 10 d of experiment A, we recorded the date of seedling emergence for each pot prior to thinning. Seedling emergence was relatively synchronous within a period of ~3–5 d after planting, so this trait was not measured in experiment B and will not be considered further. For both experiments, we measured the length of the longest rosette leaf 5 wk after planting to characterize the relative differences in rosette size prior to bolting. We also recorded the number of days from when the seeds were planted to the onset of flowering for each

plant. A plant was recorded as flowering when the first three flower buds had opened fully.

Lifetime fecundity was measured after the plants had stopped flowering and had mature fruits (siliques) that were turning light brown. Fruits were counted in wk 8 for experiment A and wk 10 for experiment B. Very young fruits less than 2 cm long and located near the branch tips were not counted. To quantify the mean number of seeds per fruit, we collected a total of 20 mature fruits per line (each from a different plant) in experiment A and 25 fruits per line in experiment B in a similar manner. These fruits were sampled from the midpoint of flowering branches to avoid sampling the youngest or oldest fruits on a given plant. Each fruit was placed in a separately labeled Microfuge tube, so that all seeds could be counted when the fruit began to dehisce. To estimate the number of seeds per plant, we multiplied the number of fruits by the average number of seeds per fruit for each line.

Data Analysis

For each trait (number of seeds per plant, number of fruits per plant, number of seeds per fruit, length of the longest leaf, and days to flowering), the differences among lines were inferred using ANOVAs and Tukey's multiple comparisons (PROC GLM, SAS, ver. 9.4, SAS Institute, Cary, NC). No data transformations were needed to meet assumptions of normality and homogeneity of variance. In the model for experiment A, block (i.e., tray) was random, while line was considered fixed. We consider line as a fixed factor because we were interested in the variation that exists among our particular lines and wanted to report the differences that exist independent of the transgene category.

For experiment B, we ran two models because the experiment included more OX and EV lines, allowing for nested analyses. In the first model, we assessed the effects of cohort, blocks (nested within cohorts), and line on each measured trait. Cohorts and blocks (nested within cohorts) were random, while line was considered fixed, similar to, and for the same reasons as, the model run for experiment A. In the second model, and solely to compare the numbers of seeds per plant (our proxy for fitness), we added the transgene category (OX, EV, and none [wild-type]) as a fixed factor, and lines were nested within this category. The ANOVA was followed by Tukey's multiple comparisons (PROC GLIMMIX, SAS, ver. 9.4, SAS Institute). Cohorts, blocks (nested within cohorts), and line (nested within the transgene category) were considered random factors, while the transgene category was considered fixed. Here, we did not necessarily care about the variation among the lines but rather wanted to see the overall effect and differences due to the transgene category (OX, EV, and none [wild-type]). Each random factor was tested with a log-likelihood ratio test to determine whether its variation was significantly different from zero (Bolker et al. 2009).

Results

Total Seeds per Plant

Total lifetime seed production reflects the cumulative differences among lines over the course of each experiment. OX1

was used in both experiments and had consistently high seed production relative to wild-type and EV lines. In experiment A, OX1 produced 35% more seeds per plant than wild-type plants, while EV3 produced 24% fewer seeds (fig. 2A; ANOVA: $F = 4.08$, $P \leq 0.0001$). Differences among other lines in experiment A were not statistically significant. Experiment B included additional OX and EV lines, greater sample sizes to improve statistical power, and better nutrient availability. In experiment B, OX1 and OX2 produced significantly more seeds per plant compared with the wild-type (37% and 23%, respectively; fig. 2B; ANOVA: $F = 5.53$, $P \leq 0.0001$). Lines OX1 and OX2 also produced significantly more seeds per plant than any of their EV counterparts (EV1–7), and EV7 produced 33% fewer seeds per plant than the wild type.

For experiment B, a nested ANOVA and Tukey tests for the number of seeds per plant showed significant differences among transgene categories, with OX plants having greater fecundity compared with either wild-type plants or EV lines, which did not differ from each other (table 2). We also analyzed the full data set including line EV7, which had unusually low fecundity compared with the other EV lines, and obtained similar results. As a group, the six OX lines produced 21% more seeds per plant than EV lines 1–6 and 26% more seeds per plant than EV lines 1–7 (means in table 2).

Fruits per Plant

In experiment A, the only lines that were significantly different from each other in terms of fruit number were OX1, which produced more fruits per plant than the wild-type line, and EV3, which had fewer fruits than the wild type (fig. 3A; ANOVA: $F = 3.81$, $P \leq 0.0001$). In experiment B, OX1 produced significantly more fruits than wild-type plants, and OX2 had more fruits than EV1–3 and EV5–6 (fig. 4A; ANOVA: $F = 3.86$, $P \leq 0.0001$). EV7 stood out in having as many fruits as OX1 and OX2 but with fewer seeds per fruit.

Seeds per Fruit

In experiment A, the average number of seeds per fruit was 50.4 ($N = 180$; all data combined) and no differences were seen among lines (fig. 3B; $F = 0.93$, $P = 0.57$). In contrast, the number of seeds per fruit differed strongly among lines in experiment B with respect to EV7, which had 40% fewer seeds per fruit than wild-type plants (wild-type fruits had an average of 45.7 seeds; fig. 4B; $F = 9.19$, $P \leq 0.0001$). Smaller differences among lines were seen in OX1 and OX2, which had significantly more seeds per fruit than EV4 and EV7 (fig. 4B).

Days to Flowering

Wild-type plants started flowering at 39 d after planting (DAP) on average in experiment A versus 51 DAP in experiment B. In both experiments, significant differences were seen among lines in the onset of flowering (table 3; experiment A: $F = 3.45$, $P \leq 0.0001$; experiment B: $F = 6.29$, $P \leq 0.0001$). In experiment A, OX1 flowered ~1–3 d earlier than OX4–6 and the four EV lines. In experiment B, both OX1 and OX2 flowered ~1–3.5 d earlier than the EV lines. Lines OX1 and OX2 also flowered earlier than the wild-type plants in experi-

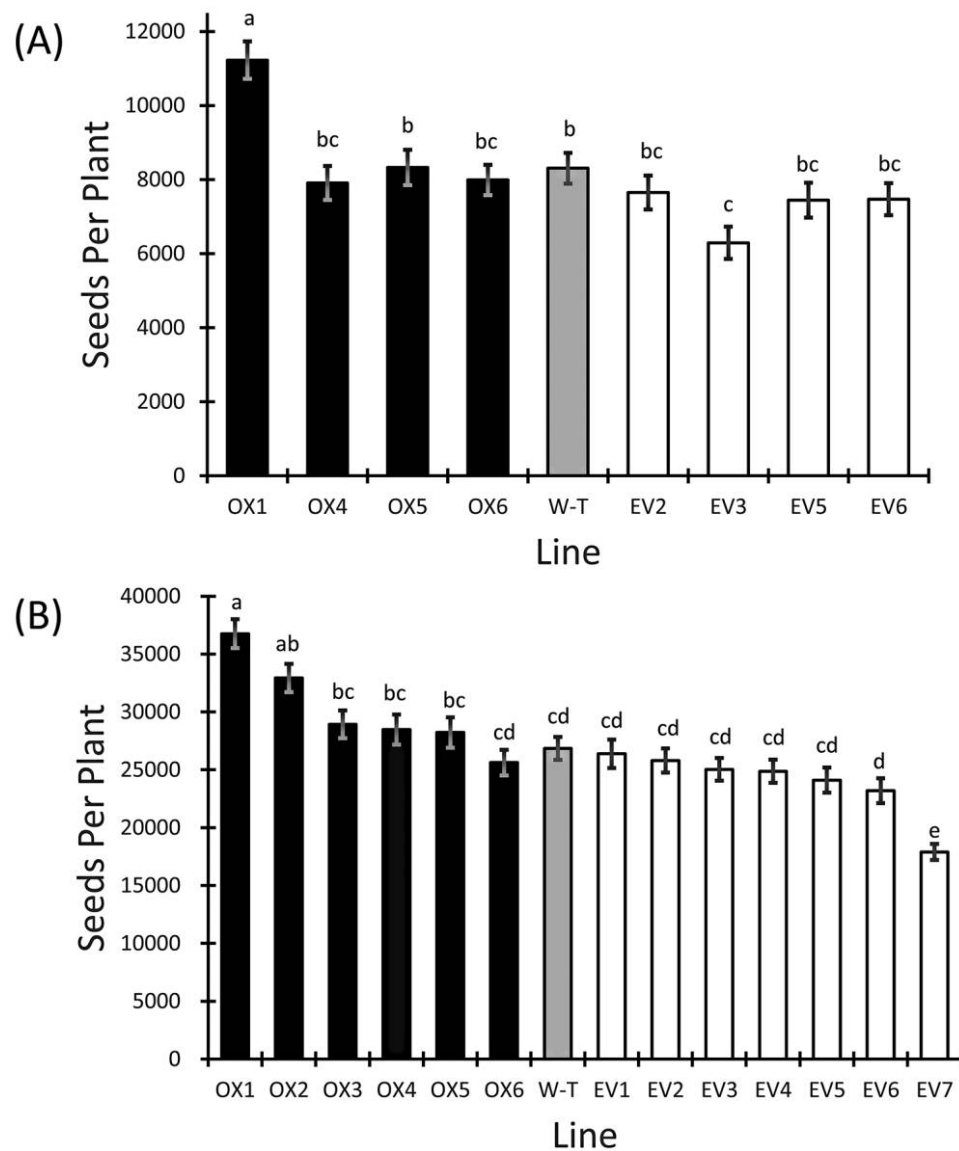


Fig. 2 Number of seeds per plant for *EPSPS* overexpression (OX), empty vector (EV), and wild-type *Arabidopsis* lines in experiments A and B, respectively. Means \pm 1 SE are displayed. $N = 45\text{--}42$ plants per line. Means that do not share superscripts are significantly different at $P \leq 0.05$ (Tukey tests).

ment B, which, in turn, flowered earlier than EV5 and EV6. Thus, OX1 and OX2 stood out as flowering earlier than all other lines, in both experiments, except for the wild type in experiment A (table 3).

Rosette Size

The length of the longest leaf was used to characterize the relative differences in rosette size prior to bolting. Plants in experiment A had shorter leaves after 5 wk than those in experiment B, with wild-type means of 59 mm versus 64 mm, respectively (figs. 3C, 4C). Modest but statistically significant differences were detected among lines in the size of 5-wk-old plants in both experiments (experiment A: $F = 2.32$, $P \leq$

0.0001; experiment B: $F = 4.87$, $P \leq 0.0001$). Two EV lines had shorter leaves than wild-type plants (experiment A, EV3; experiment B, EV6). In contrast, none of the OX lines differed significantly from wild-type plants, and two of them (OX1 and OX2) were larger than several of their EV counterparts (experiment A: EV3; experiment B: EV1–7).

Discussion

Main Effects of the Transgenes and Differences among Lines

Our results demonstrate that overproduction of EPSPS did not result in a fitness cost, based on the number of seeds per

Table 2
Nested ANOVAs for Main Effects of the OX Transgene versus Wild-Type versus Empty Vector Transgene Categories on the Number of Seeds per Plant in Experiment B

	df	Variance component	–2Res log like	χ^2	P
Random effects:					
Line (transgene)	1	7,602,391	19,613	45.26	<.0001
Cohort	1	704,452	19,568	.46	.4958
Block (cohort)	1	12,874,458	19,624	56.22	<.0001
	df (num, den)	F	P		
Fixed effects:					
Transgene	2, 10	4.78	.0349		
	Mean seeds per plant	SE	Tukey groups		
Transgene:					
EPSPS overexpression	30,160	530	A		
None (wild type)	26,408	952	B		
Empty vector (without EV7)	24,901	438	B		
Empty vector (with EV7)	23,966	405	B		

Note. Lines were nested within the transgene category (OX, none [wild type], and EV) and blocks were nested within cohort. These analyses were carried out without versus with line EV7, which had unusually low fecundity (figs. 2, 4). Similar results and Tukey groups were obtained in both cases. Tukey groups that do not share the same letter were significantly different at $P \leq 0.05$. Means and 1 SE are shown for each transgene category. num = numerator; den = denominator.

plant, and might be associated with a substantial fitness benefit. We obtained similar results from the two experiments, in that line OX1 produced the most seeds per plant and none of the other OX lines had fewer seeds than wild-type plants. Experiment B included more lines per transgene category, allowing a nested ANOVA in which the main effect of the transgene category on fecundity was statistically significant. Therefore, we conclude that the OX lines were superior to both the wild-type and EV control lines in terms of seed production (table 2; Tukey groups). In particular, two of the six transgenic lines that overexpressed *EPSPS* in experiment B, OX1 and OX2, produced significantly more seeds per plant than wild-type or EV lines (37% and 23% more seeds than wild type, respectively).

An important feature of our study was substantial variation in fitness-related traits among lines with the same OX or EV transgene. For example, line EV7 had unusually low numbers of seeds per fruit (fig. 4B), a trait that is usually considered to be relatively consistent in *Arabidopsis* (Mauricio et al. 1997). Variation in phenotypic traits among independent lines is common in studies of transgenic plants and can be found even after obviously abnormal lines have been discarded from a given experiment (Jackson et al. 2004; Yang et al. 2017a). This variation can result from position effects of transgene insertion, occurrence of a transgene multimer at the insertion site, and/or mutations generated by *Agrobacterium* at either the transfer DNA insertion site or off-target sites during the production of T1 plants. Thus, in studies where variation among transgenic lines is observed, multiple lines per construct are needed to (1) allow for statistical procedures that test for the main effects across lines (e.g., nested ANOVAs) and/or (2) obtain more than one transgenic event to support a given hypothesis (Jackson 2004; Jin et al. 2017).

Here, variation in levels of *EPSPS* expression among the OX lines raises additional questions about the relationship be-

tween the expression level of *EPSPS* and the effects on plant growth and reproduction. Three OX lines (OX4, OX5, and OX6) had greater levels of *EPSPS* gene expression and glyphosate resistance, compared with OX1, OX2, and OX3 (fig. 1B; Yang et al. 2017a), but their fecundity was not significantly different than wild-type plants (fig. 2B). This finding might indicate a cost of greater levels of overexpression, but OX3 did not fit this pattern and the small number of OX lines (six) precludes a definitive conclusion about how intermediate versus greater levels of *EPSPS* affect fecundity in *Arabidopsis*.

To reiterate, a major finding of this study is that overproduction of *EPSPS* was associated with greater seed production in two of the six transgenic OX lines. It is possible that these two lines had enhanced growth and reproduction by chance. For example, perhaps genetic modifications arising from the process of *Agrobacteria*-mediated transformation stimulated growth in these particular lines. However, we think an alternative hypothesis that warrants further investigation is that overproducing *EPSPS* had direct effects on downstream metabolic reactions that stimulated growth. This hypothesis is consistent with several related studies and could have important implications for the performance of glyphosate-resistant weed biotypes in the field. First, Wang et al. (2014) studied an OX transgene with a ubiquitin promoter from *Zea mays* fused to an endogenous *EPSPS* gene from cultivated rice (*Oryza sativa*). When this single transgenic event was crossed with conspecific weedy rice accessions, F_2 progeny that inherited the transgene had greater levels of tryptophan production and 48%–125% more seeds per plant compared with control lines (Wang et al. 2014). Second, increases in fecundity occurred when this same OX transgene from rice was transferred to wild rice (*Oryza rufipogon*) via hand pollination (Yang et al. 2017b). Furthermore, Fang et al. (2018) reported similar increases in fecundity when the same *EPSPS* gene from rice and two other *EPSPS* genes each were inserted into three indepen-

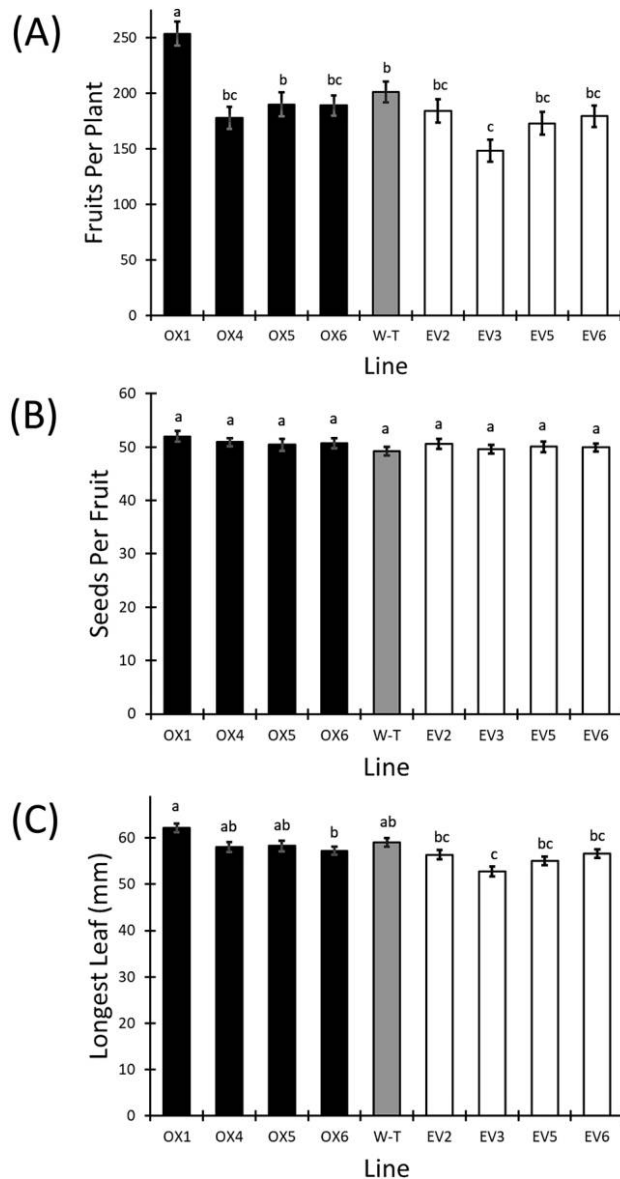


Fig. 3 Experiment A: fruits per plant (A), seeds per fruit (B), and length of the longest rosette leaf at week 5 (C) for *EPSPS* overexpression (OX), empty vector (EV), and wild-type *Arabidopsis* lines. Means \pm 1 SE are displayed. $N = 45$, except for seeds per fruit ($N = 20$). Means that do not share superscripts are significantly different at $P \leq 0.05$ (Tukey tests).

dent transgenic lines of *Arabidopsis*. Together, these studies support our hypothesis that transgenic overexpression of *EPSPS* can lead to greater fecundity in *Arabidopsis*. Additional research is needed to test the generality of these results in a broader range of species, transgenic events, and environmental conditions. In future studies involving transgenic lines that overexpress *EPSPS*, gene insertion/deletion via CRISPR/Cas9 or similar techniques will offer advantages over *Agrobacterium*-mediated transformation (Cermak et al. 2017).

Fitness Implications for Weeds That Overproduce EPSPS

Extrapolating from studies of transgenic *Arabidopsis* to glyphosate-resistant weeds is admittedly speculative, but findings based on this model species provide a starting point for understanding how surplus *EPSPS* may affect plant growth and reproduction. Several species listed in table 1 became resistant to field levels of glyphosate (i.e., ~ 840 kg acid equivalent ha^{-1}) with only two or three extra copies of *EPSPS*, while others have acquired ~ 10 – 100 copies. In all of these species, resistant genotypes exhibit increased *EPSPS* gene expression and *EPSPS* protein levels in the absence of glyphosate (references in table 1). *EPSPS* overexpression may have complex associations with the expression of other genes (Pline-Srnic 2006; Maeda and Dudareva 2012), including genes coding for phosphofructokinase and glutathione transferase (Chen et al. 2017). Carrot cell lines that overproduced *EPSPS* had higher levels of free amino acids than normal cell lines (Nazfiger et al. 1984), while Wiersma et al. (2015) found no differences in transcript levels of chorismate synthase, downstream from *EPSPS*, in glyphosate-resistant *Kochia scoparia*. Given the complexity of how various genes, transcription factors, and feedback loops interact in the shikimate pathway and throughout the plant's life cycle, additional studies at the whole-plant level are needed to test for the effects of different basal levels of *EPSPS* activity on plant performance.

Results from the current study suggest that overproduction of *EPSPS*, per se, may not confer a fitness penalty in weedy species and could be associated with a fitness benefit under certain conditions, as proposed by Wang et al. (2014). To date, empirical studies of glyphosate-resistant weeds with *EPSPS* amplification offer mixed evidence for an associated fitness penalty and no evidence for a benefit. For example, two studies of *Amaranthus palmeri* did not detect fitness effects due to *EPSPS* amplification (Giacomini et al. 2014; Vila-Aiub et al. 2014), while a fitness penalty was found in *Lolium perenne* (Yannicari et al. 2016), *Amaranthus tuberculatus* (Wu et al. 2017), and some genotypes of *K. scoparia* (Martin et al. 2017; but not in Kumar et al. 2015). Because all of these studies were carried out in the greenhouse, similar to our work with *Arabidopsis*, additional research is needed to test whether consistent results can be obtained under field conditions.

In conclusion, knowledge of the fitness effects associated with herbicide resistance is useful to understand the evolutionary dynamics of resistance traits, but estimating such effects has been challenging. With regard to overexpression of *EPSPS*, further research involving different levels of expression in a model system like *Arabidopsis*, as well as in weed species that have evolved this trait, will be informative. From the literature to date (Délye et al. 2013; Wu et al. 2017), well-designed studies are beginning to show that various types of herbicide resistance do not necessarily confer underlying fitness costs, even at high levels of resistance to a particular herbicide. If, as observed in our study, traits selected for by herbicide resistance prove beneficial in the absence of the herbicide, these traits will likely persist and theoretically even spread relative to preselection traits within wild populations. In this scenario, and also when no fitness penalty occurs, options for herbicide resistance management are limited, and an arms race ensues as weeds

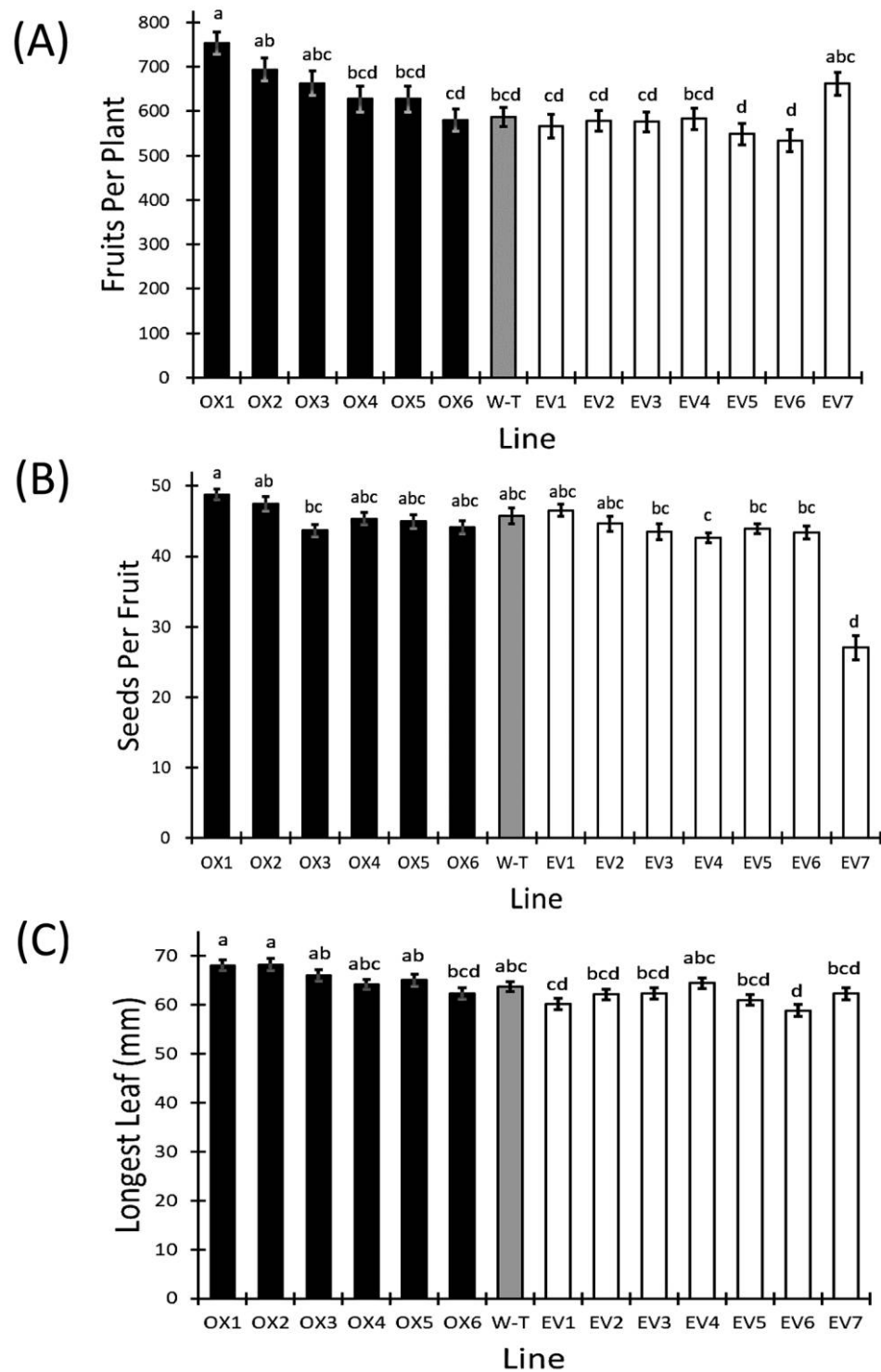


Fig. 4 Experiment B: fruits per plant (A), seeds per fruit (B), and length of the longest rosette leaf at week 5 (C) for *EPSPS* overexpression (OX), empty vector (EV), and wild-type *Arabidopsis* lines. Means \pm 1 SE are displayed. $N = 66-72$, except for seeds per fruit ($N = 22-25$). Means that do not share superscripts are significantly different at $P \leq 0.05$ (Tukey tests).

Table 3
Number of Days to Flowering for Experiment A and Experiment B for OX,
Empty Vector, and Wild-Type Lines of *Arabidopsis thaliana*

Line	Experiment A				Experiment B			
	Mean days to flowering	N	SE	Group	Mean days to flowering	N	SE	Group
OX1	37.7	45	.3	a	49.0	72	.2	a
OX2	-	-	-	-	49.0	71	.3	a
OX3	-	-	-	-	50.1	69	.3	b
OX4	40.6	45	.3	c	51.7	72	.2	defg
OX5	40.0	45	.3	bc	50.9	72	.3	bcde
OX6	39.7	45	.3	bc	51.9	72	.2	efg
Wild-type	38.9	45	.2	ab	51.0	72	.2	bcde
EV1	-	-	-	-	51.2	72	.2	cdef
EV2	39.8	45	.3	bc	50.9	72	.2	bcd
EV3	40.9	45	.4	c	51.6	71	.3	cdefg
EV4	-	-	-	-	51.6	71	.3	cdefg
EV5	40.4	45	.3	c	52.2	70	.2	fg
EV6	39.6	45	.3	bc	52.5	72	.3	g
EV7	-	-	-	-	50.6	64	.3	bc

Note. Means and 1 SE are shown. Tukey groupings that do not share letters are significantly different at $P \leq 0.05$. Lines with dashes were not used in experiment A. OX = *EPSPS* overexpression; EV = empty vector.

become ever more resistant to multiple herbicides, thereby compounding an already urgent problem in conventional agriculture (Duke and Powles 2008; Mortensen et al. 2012; Owen 2016).

Acknowledgments

We thank Emily Ernst, Allison Guggenheimer, and Jordan Williams for technical assistance and Kristin Mercer, Steven

Hovick, Micheal Owen, Mithila Jugulam, and anonymous reviewers for helpful comments on the manuscript. This project was supported by the Weedy and Invasive Plants Grant Program, grant 2016-67013-24804, from the US Department of Agriculture National Institute of Food and Agriculture (recipient: Ohio State University, A. Snow, and D. Mackey). X. Yang was partly funded by the International Postdoctoral Exchange Fellowship Program, Office of China Postdoctoral Council.

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