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Tansley review

Do plants pay a fitness cost to be resistant to glyphosate?

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Summary

We reviewed the literature to understand the effects of glyphosate resistance on plant fitness at the molecular, biochemical and physiological levels. A number of correlations between enzyme characteristics and glyphosate resistance imply the existence of a plant fitness cost associated with resistance-conferring mutations in the glyphosate target enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). These biochemical changes result in a tradeoff between the glyphosate resistance of the EPSPS enzyme and its catalytic activity. Mutations that endow the highest resistance are more likely to decrease catalytic activity by reducing the affinity of EPSPS for its natural substrate, and/or slowing the velocity of the enzyme reaction, and are thus very likely to endow a substantial plant fitness cost. Prediction of fitness costs associated with *EPSPS* gene amplification and overexpression can be more problematic. The validity of cost prediction based on the theory of evolution of gene expression and resource allocation has been cast into doubt by contradictory experimental evidence. Further research providing insights into the role of the *EPSPS cassette* in weed adaptation, and estimations of the energy budget involved in *EPSPS* amplification and overexpression are required to understand and predict the biochemical and physiological bases of the fitness cost of glyphosate resistance.

I. Introduction

1. Glyphosate resistance evolution

Weed infestations are a persistent constraint on the economy and productivity of grain cropping systems (Oerke, 2006). Since their initial introduction 70 yr ago, synthetic herbicides have successfully enhanced global food production by reducing weed densities in agroecosystems (National Research Council, 2000; Powles, 2008, 2014). The use of a particular herbicide, glyphosate, substantially increased after the first commercial release of engineered glyphosate-resistant crops in 1996 (Duke & Powles, 2008). Today,

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glyphosate has become the most widely used herbicide in global agriculture (James, 2016), with 181 million ha of transgenic glyphosate-resistant crops under cultivation (Duke, 2018).

When considering the millions of hectares of cropped land infested by billions of weed plants that are under recurrent glyphosate treatment, it is likely that the strongest selection pressure on weeds in agroecosystems is exerted by glyphosate (Palumbi, 2001; Neve *et al.*, 2009). This has inevitably led to glyphosate resistance evolution in an ever-growing list of weed species (Powles & Yu, 2010; Sammons & Gaines, 2014; Heap, 2018). Given the global importance of glyphosate and the explosion of glyphosate resistance in weeds from several major crop regions here, we concentrate on glyphosate resistance and fitness cost. We also concentrate on resistance at the glyphosate target-site enzyme, plastidic 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS). One of the target-site-based EPSPS glyphosate resistance mechanisms is the result of random DNA mutations in the *EPSPS* gene (Box 1), permitting survival and reproduction despite glyphosate treatment.

Detailed studies on the biochemical and molecular mechanisms that can be responsible for glyphosate resistance are reviewed elsewhere (Powles, 2008; Preston & Wakelin, 2008; Shaner, 2009; Powles & Yu, 2010; Sammons & Gaines, 2014). Briefly, field-evolved glyphosate resistance in weed species can be caused by target- (EPSPS) and/or nontarget-site mechanisms. Whereas the target-site resistance mechanisms involve mutation, amplification and/or overexpression of the *EPSPS* gene, the nontarget-site resistance mechanisms documented thus far include reduced leaf uptake and translocation of glyphosate. Enhanced vacuolar sequestration of glyphosate is quite a common resistance mechanism reported in many resistant weed species. Additionally, a recently reported novel resistance mechanism involves rapid tissue necrosis by as-yet-unknown mechanisms that limit glyphosate transport in resistant *Ambrosia trifida* (Moretti *et al.*, 2018). It is important to realize that both target- and nontarget-site glyphosate resistance mechanisms can coexist within an individual plant and within plant populations (Bostamam *et al.*, 2012; Morran *et al.*, 2018). Thus, individual plants and/or populations can express both different target-site (e.g. EPSPS mutation and amplification) (Chen *et al.*, 2015) and/or nontarget-site (e.g. reduced leaf uptake and translocation) glyphosate resistance mechanisms (Vila-Aiub *et al.*, 2012).

Whereas our understanding of nontarget-site glyphosate resistance mechanisms has increased in recent years, more is known about the target-site resistance mechanisms, and at a deeper level (Salas *et al.*, 2012; Jugulam *et al.*, 2014; Nandula *et al.*, 2014; Sammons & Gaines, 2014; Chatham *et al.*, 2015; Wiersma *et al.*, 2015; Malone *et al.*, 2016; Ngo *et al.*, 2018). Therefore, target-site EPSPS-based glyphosate resistance is the subject of our analysis in this paper.

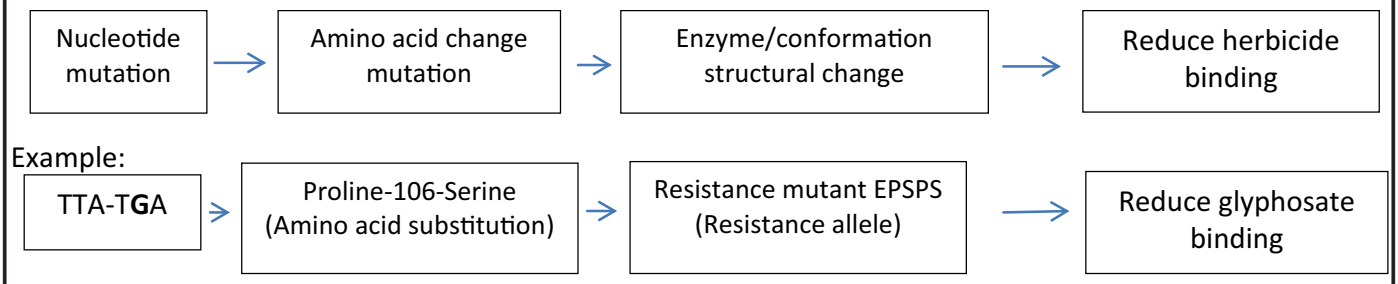
2. Herbicide resistance genes are rare traits in herbicide-free environments

Herbicide resistance is the ultimate example of the extraordinary capacity of weeds to evolve under stressful conditions (Neve *et al.*, 2009, 2014; Powles & Yu, 2010). Herbicide resistance alleles are rare in herbicide=-unselected weed populations (Preston & Powles, 2002; Neve & Powles, 2005; Busi *et al.*, 2012). A number of processes could account for this. The low frequency of resistance alleles in herbicide-unselected weed populations might reflect the low mutation rate of the gene in question and the long generational time required for new resistance allele(s) to be fixed in large populations (Kimura, 1962, 1970). Genetic drift may also lead to the loss of these rare resistance genes, especially in small populations (Kimura & Ota, 1969). Third, central evolutionary biology principles predict that adaptation (in this case, herbicide resistance) often is not cost-free (Fisher, 1928, 1958; Herms & Mattson, 1992; Bergelson & Purrington, 1996). Thus, logically, herbicide resistance evolution does not occur in herbicide-free environments (Holt & Thill, 1994; Bergelson & Purrington, 1996; Vila-Aiub *et al.*, 2009b, 2011), especially if there is selective disadvantage (s) (i.e. fitness (W) cost) experienced by resistant (R) vs susceptible (S) individuals ($s = 1 - (WR/WS)$) (Gillespie, 1998). Estimation of both genetic drift and fitness cost is central to understanding the equilibrium frequencies of herbicide resistance alleles in environments without herbicide selection.

3. How common are fitness costs of herbicide resistance genes?

Contrary to the often-reported fitness costs associated with antibiotic and insecticide resistance (Andersson & Hughes, 2010;

Box 1 A resistance gene is caused by a DNA nucleotide mutation leading to an amino acid substitution in the gene product (herbicide target enzyme). This amino acid substitution leads to a structural conformation change in the target enzyme that minimizes herbicide binding.



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Kliot & Ghanim, 2012; Melnyk *et al.*, 2015), many studies have shown no fitness tradeoffs associated with herbicide resistance genes in weeds. Our meta-review, conducted a decade ago, showed that herbicide fitness costs do not always occur, as their expression depends on the particular resistance gene, allele and genetic background (Vila-Aiub *et al.*, 2009b). One well-known case of herbicide resistance imposing a fitness cost is target-site resistance to herbicides inhibiting photosynthesis (triazine herbicides). Often, and globally, studies routinely identify in many species and environments that a single nucleotide mutation of the photosynthetic *psbA* gene changes serine at position 264 to glycine (Ser-264-Gly) (Gronwald, 1994). The chloroplastic, plastid-encoded *psbA* gene encodes the D1 protein, an essential component of the photosynthetic photosystem II (PSII) electron transfer complex. Thus, the Ser-264-Gly mutant allele endows resistance to triazine and certain other PSII-inhibiting herbicides. It has been widely observed that plants with the resistance-endowing Ser-264-Gly allele express a mean fitness cost of 25% (Gronwald, 1994; Bergelson & Purrington, 1996; Darmency, 2013).

4. Can herbicide resistance fitness costs be predicted?

A fitness cost is the adverse impact of a herbicide resistance allele on the survival and/or reproduction of resistant plants that reduces their frequency compared with the frequency of plants without resistance alleles (Cousens & Fournier-Level, 2018). A fitness cost integrates all of the genetic, biochemical and physiological changes driven by a particular resistance gene interacting within a particular genetic and ecological background. A brief examination of the fitness cost associated with the well-known triazine herbicide resistance-endowing Ser-264-Gly *psbA* gene allele can provide insights into how to potentially predict fitness costs associated with glyphosate resistance genes.

Photosystem II-inhibiting triazine and other herbicides block electron transfer by competitively displacing plastoquinone Q_B (PQ_B) from its binding site at the D1 protein. The N atom at the ethylamino residue of atrazine forms strong H-bonds to the hydroxyl group of the Ser-264 amino acid in the binding site at the D1 protein (Tietjen *et al.*, 1991). However, when the Ser-264 residue, which interacts directly with the atrazine molecule, is substituted by 264-Gly in the D1 protein, atrazine binding is weakened due to loss of H-bonds, and resistance is the result. The Ser-264-Gly mutant D1 protein is still functional but has reduced PQ_B affinity, reducing photosynthetic electron transfer and thus photosynthesis rate, which causes the observed fitness tradeoff in triazine-resistant plants (reviewed in Gronwald, 1994; Holt & Thill, 1994; Devine & Shukla, 2000). This fundamental understanding of the molecular and biochemical consequences of the Ser-264-Gly mutant allele helps us to interpret the origin of the fitness cost associated with this mutation (Gronwald, 1994). By extrapolating this comprehensive approach to other herbicide resistance gene mutations, it is possible that our current knowledge on the molecular biology of resistance genes will help to anticipate the probable expression of fitness costs (Coustau *et al.*, 2000; Vila-Aiub *et al.*, 2009b; ffrench-Constant & Bass, 2017).

II. Scope of this review

In recent years there has been substantial progress in elucidating the molecular and biochemical bases of herbicide resistance mechanisms (Patzoldt *et al.*, 2006; Iwakami *et al.*, 2012, 2014; Cummins *et al.*, 2013; Gaines *et al.*, 2014; Goggin *et al.*, 2016; Chu *et al.*, 2018; LeClere *et al.*, 2018). Some of this progress has been possible due to advances in genomics and transcriptomics technology (Ravet *et al.*, 2018) that help to identify novel target- (LeClere *et al.*, 2018) and nontarget-site resistance genes (Peng *et al.*, 2010; Yuan *et al.*, 2010; Gaines *et al.*, 2014; Délye *et al.*, 2018). Studies on the molecular biology and physiology of glyphosate resistance in several weed species have contributed to a broader and deeper understanding of herbicide resistance evolution. For evolved glyphosate-resistant weeds, resistance mechanism studies reveal *EPSPS* gene amplification (Gaines *et al.*, 2010a; Jugulam *et al.*, 2014; Patterson *et al.*, 2017) through the inheritance of replicating extrachromosomal circular DNA molecules (Koo *et al.*, 2018a,b), *EPSPS* transcriptional regulation (Zhang *et al.*, 2018), EPSPS double mutants (Funke *et al.*, 2009; Sammons & Gaines, 2014; Chen *et al.*, 2015, 2017; Yu *et al.*, 2015; Sauer *et al.*, 2016; Hummel *et al.*, 2018; Sammons *et al.*, 2018) and vacuolar sequestration of glyphosate via ABC transporters, with the dependence of this process on light (Sharkhuu *et al.*, 2014) and temperature (Ge *et al.*, 2014). The substantial research effort that continues to reveal glyphosate resistance mechanisms/mutations reflects that glyphosate is the most globally used herbicide and highlights the intriguing evolutionary pathways used by weed species to resist glyphosate.

Importantly, the equilibrium frequency of such glyphosate resistance-endowing alleles in the landscape depends on whether or not the specific resistance mechanism imposes a fitness cost (Vila-Aiub *et al.*, 2009b, 2011). Our objective here is to examine the possible detrimental effects of glyphosate resistance-endowing alleles on plant fitness traits. To achieve this goal, we first summarize fitness cost mechanisms at the biochemistry level to provide a theoretical framework for the broad prediction of fitness costs in herbicide-resistant plants. Second, we review the current understanding of the impact of glyphosate resistance alleles on plant biochemistry and physiology. Finally, our theoretical predictions of glyphosate fitness costs are compared with empirical results from published studies.

III. Herbicide resistance fitness costs at the biochemistry level

Understanding the likely effects of herbicide resistance genes/alleles on plant biochemistry and metabolism is essential to predict the expression of a resistance fitness cost (ffrench-Constant & Bass, 2017; Cousens & Fournier-Level, 2018). Resistance costs must be understood within a solid conceptual framework of plant biochemistry and evolutionary ecology. Therefore, for glyphosate resistance, we describe here the theoretical mechanisms behind the fitness costs associated with glyphosate resistance.

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1. Costs imposed by impaired enzyme catalytic activity

With few exceptions, herbicides are toxic to plants by inhibiting enzymes with essential roles in plant metabolism (reviewed in Powles & Yu, 2010). Gene nucleotide mutation causing specific amino acid substitution in a herbicide target-site enzyme causes change in the geometry of the target enzyme, reducing or even eliminating effective herbicide binding and thus conferring herbicide resistance at the whole-plant level (Schönbrunn *et al.*, 2001; Zhang *et al.*, 2004; McCourt *et al.*, 2006). If the resistance mutation compromises the normal catalytic activity of the target-site enzyme, then changes in metabolism and plant performance might occur. Depending on the degree of the catalytic activity change and the potential for compensation from other metabolic pathways, a reduction in plant fitness could result. Changes in enzyme catalytic capacity such as reaction velocity (expressed as the rate of the catalysed reaction under saturating substrate concentrations, V_{max}) and substrate affinity (expressed as the Michaelis constant, K_m, which is the substrate concentration required for a reaction to proceed at 50% V_{max}) are expected to affect the amounts of enzyme products with potential detrimental effects on plant fitness. As mentioned earlier, for instance, reduced PQ_B binding by the Ser-264-Gly D1 protein diminishes the efficiency of electron transfer in PSII, reducing photosynthesis and thus fitness (Gronwald, 1994; Holt & Thill, 1994).

2. Costs imposed by increased energy requirements for gene amplification/overexpression

Avenues to increase the amount of gene products in high demand include gene amplification or overexpression (Stark & Wahl, 1983). The increase in gene products seen, for example, in glyphosate resistance endowed by amplification or overexpression of the *EPSPS* gene necessarily involves material and energy costs, and this could be a limiting factor for cell division and proliferation (Lynch & Marinov, 2015). Theoretically, the selective advantage of a gene whose dosage has been modified in response to an environmental pressure will depend on the change in the overall cell energy budget required for gene duplication, transcription and translation processes (Wagner, 2005; Lynch & Marinov, 2015).

The structural cost of a gene involves energy expenditure in the form of ATP and phosphate hydrolysis. At the gene duplication level, processes such as nucleotide synthesis, DNA double helix unwinding, ligation and extension, and nucleosome synthesis are energy-requiring (Lynch & Marinov, 2015). At the transcriptional level, ribonucleotides and mRNA synthesis involve an energy cost, which depends on mRNA number and intron length, and on transcript turnover rate. At the protein level, synthesis of tRNA, ribosomes and amino acids, as well as protein elongation, demand substantial investment in carbon (C), nitrogen and cell energy (Akashi & Gojobori, 2002; Barton *et al.*, 2010). In the yeast *Saccharomyces cerevisiae*, duplication of a gene at the mRNA and protein levels incurs an extra material and energy cost which is high enough to be selected against by the environment (Wagner, 2005).

A classic example of a fitness cost through cell energy expenditure is provided by *Escherichia coli*. C and ATP acquisition in *E. coli* can be provided by lactose metabolism. In an environment without lactose, this metabolism is repressed at the transcriptional level (Beckwith & Zipser, 1970; Dykhuizen & Davies, 1980). *E. coli* strains unable to repress lactose metabolism exhibit constitutive energy costs due to the continual transcription and translation of a particular lactose-hydrolysing gene (Stoebel *et al.*, 2008). These metabolic costs are proportional to gene size and amount of protein produced and have detrimental effects on population growth.

For glyphosate resistance endowed by *EPSPS* gene amplification, the energy expenses involved in achieving higher levels of plant *EPSPS* gene expression/amplification may also impose constraints on fitness and selection in glyphosate-free environments where these extra gene products are unnecessary. The fundamental question posed by the resource allocation theory relates to the interaction of the resource requirements for growth vs defence (in this case, defence against glyphosate via gene amplification or overexpression). In essence, will growth be limited by the availability of materials and energy, due to extra investment in the synthesis of defensive gene products? (reviewed in Herms & Mattson, 1992; Bergelson & Purrington, 1996). A good example from insecticide resistance is that amplification of detoxifying esterase genes is often found as the mechanism conferring resistance to organophosphorus insecticides in mosquitoes, arthropods and aphids (Raymond *et al.*, 1998; Paton *et al.*, 2000; Bass & Field, 2011). Mosquito strains with esterase gene amplification showed higher mortality rates (+46%) and lower lipid and sugar reserves (−20%), an indication that the C and energy load associated with this gene amplification has a fitness cost (Rivero *et al.*, 2011). One example from herbicide resistance is that variations in acetolactate synthase (ALS) activity in different *Arabidopsis thaliana* lines transformed with a herbicide-resistant *ALS* gene (a Pro-197-Ser mutant allele) corresponded positively to different amounts of synthesized free amino acids and plant fitness costs, probably due to the energy requirement for amino acid synthesis (Fig. 1) (Purrington & Bergelson, 1999).

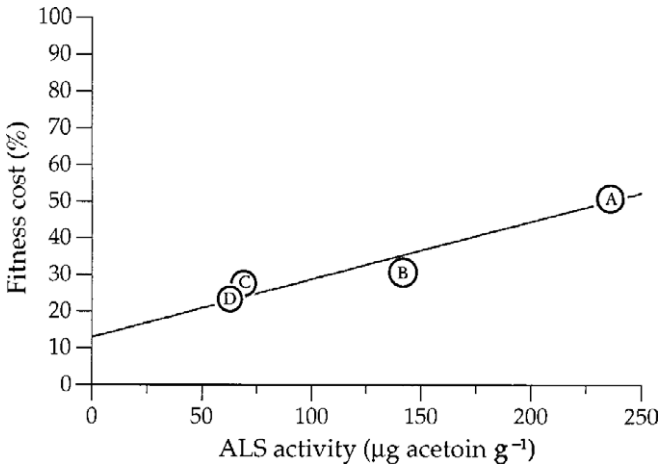

Fig. 1 Fitness cost in transgenic *Arabidopsis thaliana* plants expressing the Csr1-1 *ALS* gene corresponding to the ALS Pro-197-Ser mutation found in naturally evolved resistant weed species. Four independent transgenic ALS-resistant lines (A–D) exhibited different ALS activities which correlated ($r = 0.94$, $P = 0.07$) with different expression levels of fitness cost. (Extracted from Purrington & Bergelson (1999) with copyright permission from The University of Chicago Press.)

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IV. Herbicide resistance fitness costs mediated by ecological interactions

Some fitness costs originate from gene × environment biotic interactions and may express independently of, or in addition to, the mechanisms described earlier (Strauss *et al.*, 2002). This type of fitness cost operates when the resistance trait has pleiotropic consequences on other traits that directly or indirectly affect interacting organisms (pollinators, pathogens, competitors). For instance, synthesis of secondary compounds (glucosinolates) has been shown to increase resistance to herbivorous insects in obligately outcrossing cruciferous species (reviewed in Bennett & Wallsgrove, 1994), and it was speculated that the expression of these defence glucosinolate compounds in floral structures may have consequences for pollination. Strauss *et al.* (1999) confirmed that bees spent less time foraging in the flowers of a high-glucosinolate, beetle-resistant *Brassica rapa* ecotype, compromising the selection of this resistance trait in environments with no herbivory.

Despite the known metabolic changes that may be introduced by herbicide resistance mutations (Herms & Mattson, 1992; Bennett & Wallsgrove, 1994; Maroli *et al.*, 2015; Han *et al.*, 2017), published examples of herbicide resistance fitness costs arising from ecological interactions are rare (Vila-Aiub *et al.*, 2009b). One such example is that plants with impaired photosynthesis due to the *psbA* mutation (Ser-264-Gly) have higher leaf N concentrations (Arntz *et al.*, 2000; Gassmann, 2005) and thus suffer greater beetle grazing herbivory (Gassmann & Futuyma, 2005) (Fig. 2). Feeding preferences for the N-enriched leaves have also been shown to change between herbivore species and light environments (Gassmann, 2005).

Ecological-mediated costs may also arise from intense interplant competition for water, nutrients and light, triggering and/or amplifying the expression of herbicide resistance fitness costs (Vila-Aiub *et al.*, 2009b). The cost of mechanisms such as reduced enzymatic catalytic activity (Reboud & Till-Bottraud, 1991) and/or a constrained energy budget (Vila-Aiub *et al.*, 2009a) seem to be exacerbated in resource-limited environments.

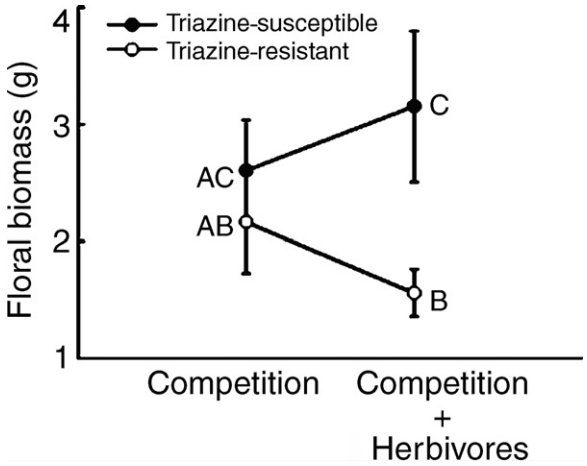

Fig. 2 Selective herbivory damage on reproductive biomass of triazine-resistant (*psbA* Ser-264-Gly mutation) *Amaranthus hybridus* from the beetle *Disonycha glabrata*. Treatments involved triazine-resistant and -susceptible plants under competition, with and without exclusion of insects under field conditions. Circular symbols represent sample means and error bars are ± SE of the mean. Letters indicate significant pairwise differences (Extracted from Gassmann & Futuyma (2005) with copyright permission from John Wiley & Sons.)

V. Should plants pay a fitness cost to be resistant to glyphosate?

Answering this question requires, first, an understanding of glyphosate mode of action and, second, an understanding of how the biochemical mechanisms that endow glyphosate resistance fit into the discussed biochemical/ecological mechanisms imposing fitness costs.

1. Interaction between glyphosate and the shikimate pathway and C flow in plants

The shikimate pathway is responsible for the biosynthesis of the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), which are only synthesized by plants, bacteria and fungi (Herrmann & Weaver, 1999; Maeda & Dudareva, 2012) and are essential building blocks for proteins, hormones (e.g. auxin) and structural and defensive phenolics (e.g. lignin, flavonoids, alkaloids) (Maeda & Dudareva, 2012). The key enzyme EPSPS catalyses the reaction which converts shikimate-3-phosphate (S3P) plus phosphoenolpyruvate (PEP) to 5-enolpyruvylshikimate-3-phosphate (EPSP), an essential step in the synthesis of chorismate, a precursor for aromatic amino acid synthesis.

In plants, the shikimate pathway is one of the most active metabolic pathways in terms of C flow (Herrmann, 1995b; Herrmann & Weaver, 1999; Tzin & Galili, 2010). Up to 30% of photosynthetically fixed C flows into the shikimate pathway, and the depletion of Phe, Tyr and Trp or their downstream products induces higher C allocation (via transcriptional and post-transcriptional regulation) to restore their normal levels (reviewed by Maeda & Dudareva, 2012). The shikimate pathway initiates from PEP and erythrose 4-phosphate, which derive from glycolysis and the pentose phosphate pathway, respectively. Some intermediates of the shikimate pathway lead to secondary metabolic processes that reversibly produce chlorogenate via its precursor, quinate. Both quinate and chlorogenate are important C sources in plants (Herrmann, 1995b): quinate is a C reservoir for biosynthesis of aromatic compounds, and chlorogenate is a disease deterrent and UV defence compound (Clé *et al.*, 2008). When glyphosate competes with PEP to bind at the catalytic site of the EPSPS–S3P complex (Schönbrunn *et al.*, 2001), consequently blocking chorismate synthesis, there is an increased C flow into the shikimate pathway via upregulation of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity (Steinrücken & Amrhein, 1980; Herrmann, 1995a). This elevated flow into the glyphosate-inhibited shikimate pathway finally results in accumulation of harmful concentrations of both quinate and shikimate in glyphosate-treated susceptible plants (Herrmann, 1995b; Geiger *et al.*, 1999; Herrmann & Weaver, 1999; Orcaray *et al.*, 2010).

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VI. Do EPSPS target-site glyphosate resistance mutations lead to impaired EPSPS activity?

Glyphosate disrupts the shikimate pathway by binding to the EPSPS catalytic site in competition with the endogenous PEP substrate (Steinrücken & Amrhein, 1980; Boocock & Coggins, 1983). A number of engineered and natural bacterial and plant EPSPS variants have been shown to prevent glyphosate binding and thus endow glyphosate resistance (Healy-Fried *et al.*, 2007; Alibhai *et al.*, 2010; Sammons & Gaines, 2014; Yi *et al.*, 2016; Sammons *et al.*, 2018). Since the first identification of a naturally evolved glyphosate resistance *EPSPS* gene mutation, resulting in a Pro-106-Ser substitution in *Eleusine indica* (Baerson *et al.*, 2002), other single amino acid substitutions (Thr, Ala, Leu) at the same Pro-106 residue have been reported to endow glyphosate resistance in weed species (Ng *et al.*, 2003; Yu *et al.*, 2007; Kaundun *et al.*, 2011; Sammons & Gaines, 2014; Morran *et al.*, 2018). Artificial and naturally evolved double *EPSPS* gene mutations have also been reported to confer glyphosate resistance in bacteria and plants (Padgette *et al.*, 1991; Kahrizi *et al.*, 2007; Funke *et al.*, 2009; Sammons & Gaines, 2014; Chen *et al.*, 2015, 2017; Yu *et al.*, 2015). The different single and double resistance-endowing *EPSPS* mutations have different effects on EPSPS catalytic activity and the amount of glyphosate resistance (Table 1). The various Pro-106 substitutions in EPSPS confer only low-level glyphosate resistance at both the enzyme and plant levels (Table 1; reviewed in Sammons & Gaines, 2014). Most studies show that mutations at Pro-106 cause only small structural changes in the EPSPS active site in bacteria and plants. The K_m values indicate that the binding affinities for PEP and S3P are unchanged (106-Ser/Gly/Ala) or slightly decreased (106-Leu) (Zhou *et al.*, 2006; Healy-Fried *et al.*, 2007; Dong *et al.*, 2019). However, the Pro-106-Leu substitution

Table 1 Known target-site 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) glyphosate resistance mutations and their effects on EPSPS kinetics and reported (and predicted) plant fitness.

Resistance mutations	$K_iR : K_iS$ ratio	*EPSPS* gene origin	EPSPS catalytic capacity: $K_{m\text{-}PEP}R : K_{m\text{-}PEP}S$ ratio	EPSPS catalytic capacity: $V_{max}R : V_{max}S$ ratio	Fitness cost[a]	References
	1000[b]	*Escherichia coli*	32		Probably high	Eschenburg *et al.* (2002)
Gly-101-Ala	5000	*Petunia hybrida*	42		Probably high	Padgette *et al.* (1991)
	29 242	*Zea mays*	35		Probably high	Dong *et al.* (2019)
Gly-101-Ser		*E. coli*	No EPSPS activity (no PEP binding)		Lethal	Padgette *et al.* (1991)
Thr-102-Ile	300	*E. coli*	8.4	0.2	Probably lethal	Funke *et al.* (2009)
	298	*Z. mays*	8.6			Alibhai *et al.* (2010)
	10.4	*Z. mays*	3.2			Dong *et al.* (2019)
Thr-102-Ser	11.3	*Helianthus annus*	3.2		Probably negligible	
	3.2[+]	*Tridax procumbens*				Li *et al.* (2018)
Pro-106-Ser	10	*E. coli*	1.6	0.4		Funke *et al.* (2009)
	14		1.2	0.4		Healy-Fried *et al.* (2007)
Pro-106-Gly	30		1.5	0.6		
Pro-106-Ala	48		1.3	0.6		Healy-Fried *et al.* (2007)
Pro-106-Leu	165		2.5 ($1.7_{(S3P)}$)	0.16		
Pro-106-Ser	21	*Eleusine indica*	2.3			Baerson *et al.* (2002)
	4.23[b]		1.0	0.9	No	Yu *et al.* (2015)

Resistance mutation	$K_iR : K_iS$ ratio	Species	EPSPS catalytic capacity: $K_{m\text{-}PEP}R : K_{m\text{-}PEP}S$ ratio	EPSPS catalytic capacity: $V_{max}R : V_{max}S$ ratio	Fitness cost	References
Pro-106-Ser	7.5	*P. hybrida*	9			Padgette *et al.* (1991)
Pro-106-Ser	5	*Z. mays*	1.2		No	Dong *et al.* (2019)
Pro-106- Leu	60		4.9		Probably negligible or low	
Pro-106-Leu	70.5	*Oryza sativa*	4.4	1.0	Probably negligible or low	Zhou *et al.* (2006)
Gly-101-Ala + Pro-106-Ser (GAPS)	20 500	*P. hybrida*	78		Probably lethal	Padgette *et al.* (1991)
Thr-102-Ile + Pro-106-Ser	8067	*E. coli*	2.2	0.12		Funke *et al.* (2009)
(TIPS)	2563[b]	*E. indica*	0.8	0.06	Very high	Yu *et al.* (2015)
Thr-102-Ile + Pro-106-Ala	148.3	*Z. mays*	14.5		Probably negligible	Alibhai *et al.* (2010)
(TIPA)	300	*Arabidopsis thaliana*				Sammons *et al.* (2018)

R : *S* ratio, resistant : susceptible ratio.
[a]See main text for discussion and references.
[b]K_iR and K_iS values estimated from the equation: $IC_{50} = K_i\,(1 + (Sub/K_{m\text{-}Sub})$ (Burlingham & Widlanski, 2003), where IC_{50} is the glyphosate inhibition constant that reduces 50% EPSPS activity, K_i is the EPSPS dissociation constant under glyphosate inhibition, 'Sub' is the substrate (phosphoenolpyruvate, PEP) concentration and $K_{m\text{-}Sub}$ is the substrate (PEP) affinity. Estimated values are from Yu *et al.* (2015).

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compromises EPSPS V_{max} by a factor of 6 in *E. coli* (Healy-Fried *et al.*, 2007) but not in rice (Zhou *et al.*, 2006) (Table 1). In addition, the Pro-106-Leu mutation also reduces EPSPS catalytic efficiency by about five-fold in maize (Dong *et al.*, 2019).

Conversely to the EPSPS Pro-106 low-level glyphosate resistance-endowing mutations, the mutation Gly-101-Ala (Gly-96 in the bacterial EPSPS numbering system) confers very high-level glyphosate resistance at the enzyme level (Table 1). However, this mutation reduces the K_m for PEP by 32 and 42 times in *E. coli* and *Petunia hybrida*, respectively (Eschenburg *et al.*, 2002). A similar result has been recently found in maize using saturation mutagenesis, where all possible amino acids are tested in a particular position (Dong *et al.*, 2019). The reduced catalytic capacity of the mutant Gly-101-Ala EPSPS enzyme is accounted for by the introduction of a methyl group into the active site which reduces binding not only of glyphosate but also of PEP (Eschenburg *et al.*, 2002). An extreme case of complete loss of EPSPS catalytic activity occurs with the substitution of highly conserved residues such as Gly-101, Lys-22 and Lys-340 in *E. coli* (Padgette *et al.*, 1991), and Arg-28 and Arg-131 in *P. hybrida* (Huynh *et al.*, 1988; Padgette *et al.*, 1988; Huynh, 1990).

The Thr-102-Ile EPSPS substitution endows moderate- to high-level glyphosate resistance in *E. coli* and maize (the inhibition constant or K_i, expressed as the concentration of glyphosate required to produce 50% maximum inhibition, is 90 μM for the Gly-101-Ala mutation and 233 μM for Thr-102-Ile, vs wild-type values of 0.3 and 0.4 μM, respectively) (Funke *et al.*, 2009; Alibhai *et al.*, 2010; Sammons & Gaines, 2014). However, the catalytic capacity of Thr-102-Ile EPSPS is reduced in terms of PEP binding (eight-fold increase in K_m) and reaction rate (five-fold decrease in V_{max}) (Funke *et al.*, 2009) (Table 1). The same Thr-102-Ile mutation in maize (K_i = 149 μM) has also been shown to increase the K_m of PEP eight-fold (Alibhai *et al.*, 2010).

Recently, a novel single Thr-102-Ser mutation of EPSPS has been reported in glyphosate-resistant *Tridax procumbens* (Li *et al.*, 2018). Structural modelling predicts that the Thr-102-Ser mutation weakly reduces glyphosate binding but enhances PEP binding (Li *et al.*, 2018). As a result, the Thr-102-Ser mutation endows a lower glyphosate resistance than the other *EPSPS* gene resistance-endowing mutations, while probably having little to no effect on EPSPS catalytic activity. This result has recently been confirmed in Thr-102-Ser EPSPS maize and sunflower lines (Dong *et al.*, 2019). The weak glyphosate binding and minimal impact on EPSPS catalytic activity may both be accounted for by the biochemical similarities between the wild-type (Thr) and the substituted resistant mutant (Ser) amino acids (Li *et al.*, 2018).

Compared with the single Pro-106 mutations discussed earlier, EPSPS double mutations exhibit significantly reduced glyphosate binding, yielding very high-level glyphosate resistance (Table 1). For example, the Gly-101-Ala/Pro-106-Ser (GAPS) or Thr-102-Ile/Pro-106-Ser (TIPS) double mutations showed dramatically increased glyphosate resistance (Padgette *et al.*, 1991; Funke *et al.*, 2009; Yu *et al.*, 2015) (Table 1). However, there are adverse consequences on the EPSPS catalytic capacities associated with these double mutations: PEP affinity and V_{max} are decreased in the GAPS and TIPS mutants, respectively (Table 1).

1. How does EPSPS activity correlate with plant fitness?

As discussed earlier, particular resistance-endowing EPSPS amino acid substitution impacts the degree of glyphosate resistance at the enzyme and plant levels. Some, but not all, of these amino acid substitutions alter EPSPS catalytic capacity (Table 1). As glyphosate competes with PEP for EPSPS binding (Boocock & Coggins, 1983) and is considered a transition state mimic of the catalysed reaction course (Schönbrunn *et al.*, 2001), the degree of glyphosate resistance depends on the extent to which the glyphosate-binding site is perturbed, whereas EPSPS catalytic activity depends on the extent to which the substrate-binding site is left intact. It is expected, then, that any resistance-endowing EPSPS mutation that significantly reduces affinity for glyphosate will also reduce affinity for PEP, resulting in a tradeoff between the resistance endowed by a mutation (K_i) and the resistance cost at the enzyme level (K_m and/or V_{max}) (Powles & Yu, 2010; Sammons & Gaines, 2014). A compilation of results from studies reporting on EPSPS target-site resistance mutations (Supporting Information Fig. S1), glyphosate resistance and EPSPS catalytic activity shows a positive correlation between the K_i for glyphosate and the K_m for PEP (Fig. 3). Similarly, there is a negative correlation between K_i and V_{max} (P = 0.005) (Fig. 4).

As outlined earlier, glyphosate resistance-endowing amino acid substitutions at Pro-106 lead only to low-level glyphosate resistance and a lack of significant changes in EPSPS functionality (Table 1). Not surprisingly, Pro-106 substitutions are the most common form of target-site glyphosate resistance (Powles & Yu, 2010; Sammons & Gaines, 2014; Morran *et al.*, 2018), and resistant individuals show no fitness cost at the plant level, and persist in populations in the absence of glyphosate selection (Yu *et al.*, 2015; Fernández-Moreno *et al.*, 2017; Han *et al.*, 2017; Wu *et al.*, 2018).

As predicted, single mutations (e.g. Gly-101-Ala, Thr-102-Ile) which endow relatively high-level resistance show greatly reduced

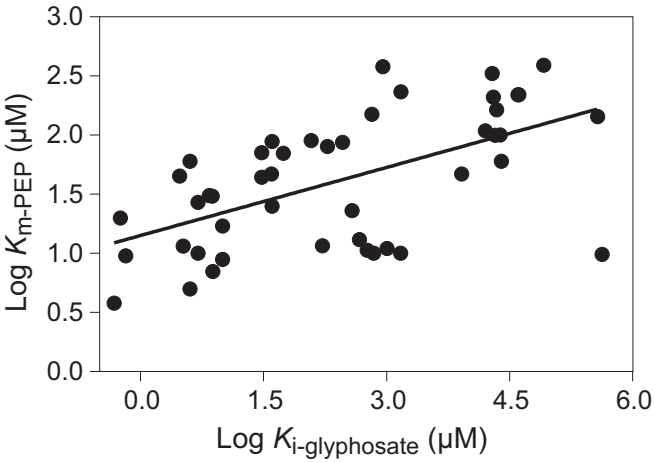

Fig. 3 Increases in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) glyphosate inhibition constant ($K_{i\text{-glyphosate}}$) are correlated ($P < 0.0001$; $R^2 = 0.33$; $n = 46$) with decreases in phosphoenolpyruvate (PEP) affinity (i.e. higher $K_{m\text{-PEP}}$ values) in a number of EPSPS glyphosate resistance mutations in bacteria and plants. Equation from regression analysis: $\log K_{m\text{-PEP}} = 1.1 + 0.192 \times ((\log K_{i\text{-glyphosate}}) + 1)$. Data are compiled from several studies (Supporting Information Fig. S1).

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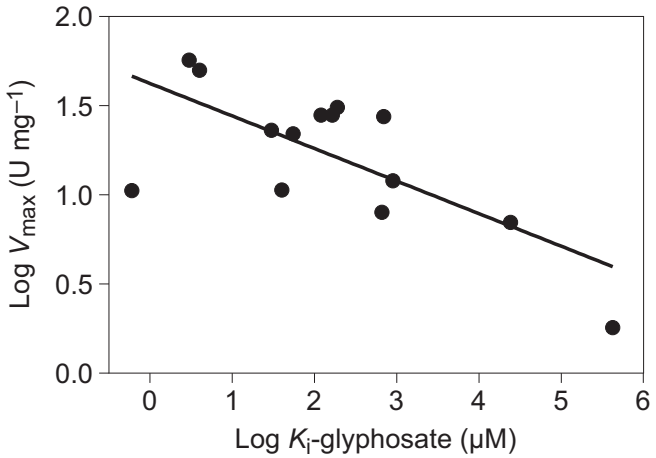

Fig. 4 Increases in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) glyphosate inhibition constant ($K_{i\text{-glyphosate}}$) are correlated ($P = 0.005$, $R^2 = 0.50$, $n = 14$) with decreases in EPSPS V_{max} in a number of EPSPS glyphosate resistance mutations in bacteria and plants. Equation from regression analysis: log EPSPS $V_{max} = 1.626 - 0.1826 \times ((\log K_{i\text{-glyphosate}}) + 1)$. Data are compiled from several studies (Supporting Information Fig. S1).

EPSPS catalytic activity when expressed in *E. coli* (Eschenburg *et al.*, 2002; Funke *et al.*, 2009; Sammons & Gaines, 2014). Thus, the evolution of glyphosate-resistant weed species possessing an EPSPS amino acid substitution at the Gly-101 residue, or a Thr-102-Ile substitution, seems unlikely. By contrast, the newly reported single EPSPS Thr-102-Ser mutation, conferring low-level glyphosate resistance in *T. procumbens*, is speculated to have no fitness cost (Li *et al.*, 2018).

The double 102/106 TIPS mutation gives much higher glyphosate resistance than the 106 mutation alone, at the same time having higher affinity for PEP than the 102 mutation alone, due to the conformational changes in TIPS EPSPS which render glyphosate binding more affected than PEP binding (Funke *et al.*, 2009; Yu *et al.*, 2015). However, this compensatory effect cannot preclude a significant reduction in catalytic activity in terms of V_{max} (Funke *et al.*, 2009; Sammons & Gaines, 2014; Yu *et al.*, 2015).

The *E. indica* TIPS double mutation endowing high-level glyphosate resistance, when expressed heterologously in *E. coli* showed only 6% of the wild-type EPSPS V_{max} (Yu *et al.*, 2015). This big reduction in V_{max} should limit the synthesis of Phe, Tyr and Trp and their downstream products and increase the amount of compensatory C flowing to the shikimate pathway (Maeda & Dudareva, 2012). Thus, this TIPS double mutation, although giving high-level glyphosate resistance, should incur a fitness penalty at the plant level. Indeed, experiments demonstrated that *E. indica* seedlings homozygous for the TIPS mutation had 20% reduction in relative growth rate compared with the wild-type, and mature plants exhibited 5% lower reproductive effort and 69% less seed number when grown without competition (Han *et al.*, 2017) (Fig. 5). A metabolic pathway analysis revealed that the reduction in growth and fecundity of the *E. indica* TIPS mutants is not associated with the depletion of aromatic amino acid pools, but rather with higher accumulation of C-rich shikimate (11-fold) and quinate (six-fold), and polar metabolites from glycolysis and carbohydrate metabolism

Fig. 5 *Eleusine indica* plants with the double Thr-102-Ile/Pro-106-Ser (TIPS) 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) glyphosate resistance mutation (upper panel) and wild-type (lower panel) growing under resource competition with rice plants. TIPS plants express a remarkable reduction in growth and fecundity that is further amplified under crop competition (Han *et al.*, 2017).

(Han *et al.*, 2017). Regulatory processes leading to the rapid turnover and degradation of protein may help to replenish the aromatic amino acid pools in TIPS mutants (Zhao *et al.*, 2018), which may come at a cost. In line with these deleterious effects of the TIPS mutation, the reported frequency of naturally evolved or CRISPR/Cas9-engineered plants with the homozygous TIPS mutation is very low or nil (Yu *et al.*, 2015; Li *et al.*, 2016).

2. Is the fitness cost of the TIPS double mutation exacerbated by ecological interactions?

Under strong competition from a rice crop, strongly glyphosate-resistant *E. indica* TIPS plants produced 85% fewer seeds compared with the wild-type (Han *et al.*, 2017). Reanalysis of data from Han *et al.* (2017) revealed that TIPS plants challenged with increasing resource competition from a rice crop show linear reductions in individual plant seed production, whereas seed production of Pro-106-Ser plants did not differ from that of wild-type plants, even at high competition intensity (Fig. 6). It is possible that the metabolic disturbance caused by the altered shikimate pathway in TIPS plants, leading to higher constitutive concentrations of C-rich shikimate and quinate, comes at a higher fitness cost in environments where light is limited by shading from a large crop canopy. This could result in a significantly lower equilibrium frequency of the TIPS mutation over generations subjected to intense resource competition without glyphosate selection.

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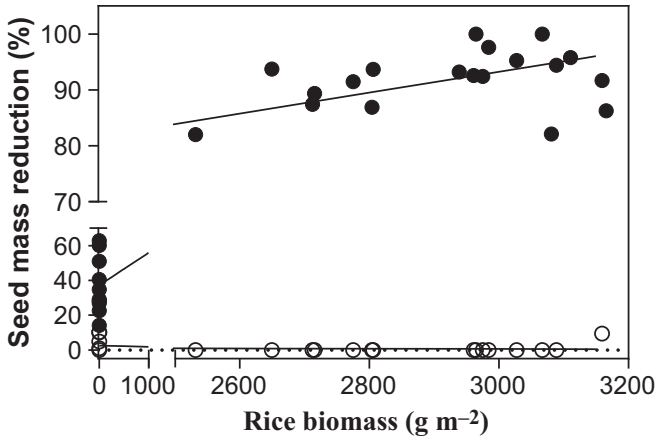

Fig. 6 Estimated reduction of reproductive output in *Eleusine indica* plants carrying the single Pro-106-Ser (open circles, $n = 25$) or double Thr-102-Ile/Pro-106-Ser (TIPS) (closed circles, $n = 29$) 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) glyphosate resistance mutations under increasing standing biomass of a rice crop. Seed mass reduction is expressed relative to the total seed mass produced by wild-type plants growing under the same conditions, after random pairwise comparisons of plants. There was a significant linear regression for the TIPS fitness cost data ($P < 0.0001$, $R^2 = 0.87$) but not for the Pro-106-Ser data ($P > 0.05$). Linear equation for TIPS data: $FC_{TIPS} = 37.2 + 0.019x$

Yu *et al.* (2015) also identified glyphosate-resistant *E. indica* plants carrying both an allele with the double TIPS mutation (designated as R) and an allele with the Pro-106-Ser mutation alone (designated as r). These compound heterozygous (Rr) TIPS plants did not express any fitness penalty, unlike the homozygous RR plants, and represented 50% of the individuals in the original field-collected *E. indica* population (Han *et al.*, 2017). However, Li *et al.* (2016) reported a 50% seed set reduction associated with true heterozygous (RS) TIPS mutant rice plants generated by CRISPR–Cas9 gene editing. The contrasting fitness effects found between the compounded (Rr) and true (RS) heterozygous EPSPS TIPS variants may be related to the degree of impact on EPSPS functionality between the r and S alleles.

A number of natural glyphosate-resistant EPSPS variants have been found in microorganisms (Barry *et al.*, 1997; Funke *et al.*, 2006; Cui *et al.*, 2016; Yi *et al.*, 2016) and engineered into crop cultivars (Barry *et al.*, 1997; Green, 2009). The basis for the commercial release of crops carrying these EPSPS variants is an acceptable degree of glyphosate resistance without substantial negative effects on EPSPS catalytic activity and, consequently, on crop yield (Funke *et al.*, 2006; Darmency, 2013; Cui *et al.*, 2016; Yi *et al.*, 2016). Recent work on *EPSPS* gene synthetic shuffling has made it possible to introduce up to 21 mutations into a single plant *EPSPS* gene to achieve glyphosate-resistant variants with near-normal catalytic functionality (Dong *et al.*, 2019).

VII. Does glyphosate resistance by *EPSPS* gene amplification and overexpression correlate with a fitness cost due to energy constraints?

Since the first identification of *EPSPS* gene amplification (Box 2) as a mechanism endowing glyphosate resistance in *Amaranthus palmeri* (Gaines *et al.*, 2010), several other evolved glyphosate-resistant weed species have been reported to possess this resistance mechanism (reviewed in Sammons & Gaines, 2014; Chatham *et al.*, 2015; Chen *et al.*, 2015; Wiersma *et al.*, 2015; Malone *et al.*, 2016; Chen *et al.*, 2017; Patterson *et al.*, 2017; Ngo *et al.*, 2018). This suggests that genetic variation for this glyphosate 'molecular sponge' mechanism is more frequent among plant species than originally anticipated (Powles, 2010; Laforest *et al.*, 2017).

In resistant plants, variations in the number of *EPSPS* gene copies positively correlates with gene expression and protein load (reviewed in Patterson *et al.*, 2017). For instance, *A. palmeri* plants with 54 *EPSPS* gene copies synthesize 20-fold more EPSPS protein than do plants with one copy (Gaines *et al.*, 2010). The *EPSPS* gene copy number ranges from two to more than 150 across and within weed species, and higher glyphosate resistance is associated with increasing EPSPS copy number (Vila-Aiub *et al.*, 2014; Gaines *et al.*, 2016). This amazing increase in *EPSPS* gene copy number (up to 150-fold) and thus overproduction of EPSPS protein must incur additional energy and material expense that should translate into a plant fitness cost in the absence of glyphosate selection. Thus far, seven studies with three glyphosate-resistant weed species (*Amaranthus palmeri*, *Amaranthus tuberculatus* and *Kochia scoparia*) have evaluated the expression of plant fitness costs associated with *EPSPS* gene amplification. Plants from different *A. palmeri* populations with constitutive *EPSPS* gene amplification yielding up to *c.* 90 copies exhibited no negative effects on plant growth and reproductive fitness traits (Giacomini *et al.*, 2014; Vila-Aiub *et al.*, 2014). Inbred *K. scoparia* individuals with two vs 14 copies of EPSPS showed no differences in fitness traits (Kumar & Jha, 2015). Another study assessed six segregating F_2 *K. scoparia* populations in which fitness was compared between individuals with low (one) vs high (10) EPSPS copy number under intraspecific competition within each population (Martin *et al.*, 2017). Overall, the effect of *EPSPS* gene amplification on plant fitness traits depended on the particular population genetic background. Plants from four populations, each with 10 *EPSPS* copies, showed no decreased fitness as compared with plants with no *EPSPS* gene amplification. In two other *K. scoparia* populations with 10 copies, however, plants showed average reductions of 70% and 75% in individual seed weight production and viability, respectively (Martin *et al.*, 2017). No fitness cost was identified in various *K. scoparia* populations from Kansas (USA) in which glyphosate-resistant individuals exhibited an average of five to six *EPSPS* gene copy numbers (Osipitan & Dille, 2019).

EPSPS gene overexpression (rather than amplification) has shown a fitness tradeoff when conferring glyphosate resistance in *Lolium perenne* (Yanniccari *et al.*, 2016, 2017). Glyphosate-resistant plants exhibiting 15-fold more EPSPS transcripts and three-fold more EPSPS activity displayed a 40% reduction in the total number of seeds produced under field conditions.

Extrapolating from Gaines *et al.* (2010), 90-fold more *EPSPS* gene amplification would represent about 40 times more EPSPS activity in glyphosate-resistant *A. palmeri* plants. Phenyalanine-derived compounds may account for up to 30% of organic matter in some plant species (Maeda & Dudareva, 2012) and the three aromatic amino acids incur, by far, the highest metabolic cost in

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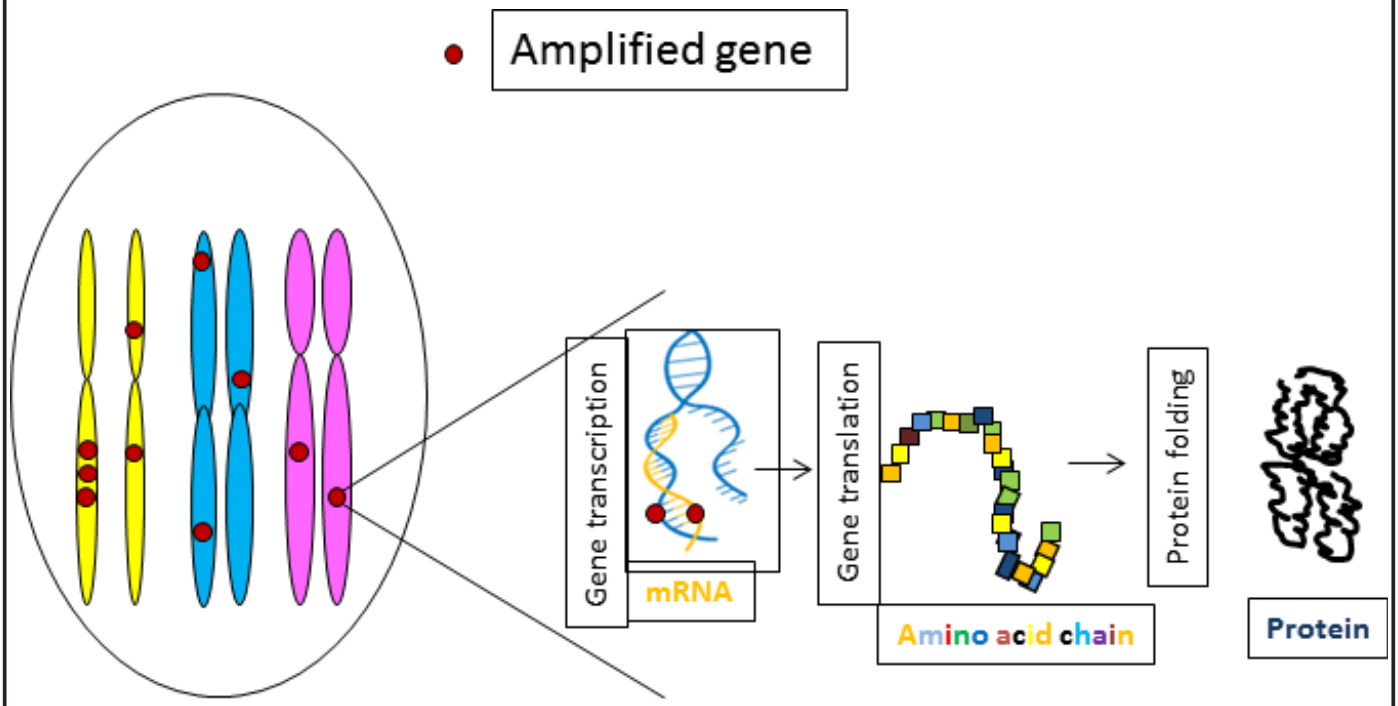
Box 2 Structural cost of an amplified gene involves extra energy expenditure in subprocesses during DNA replication and gene transcription and translation. An increase in the number of ATP and P demanding processes such as nucleotide synthesis, DNA double helix unwinding, ligation and extension and nucleosome synthesis (DNA replication), ribonucleotides and mRNA synthesis (gene transcription), and synthesis of tRNA, ribosomes, amino acids and their precursors, as well as protein elongation (gene translation) is expected as the number of amplified gene copy numbers increases.



amino synthesis in bacteria and yeast (Akashi & Gojobori, 2002; Barton *et al.*, 2010). Given the material and energy expenses involved not only in producing extra copies of the *EPSPS* gene, transcript and protein (Akashi & Gojobori, 2002; Barton *et al.*, 2010; Tzin & Galili, 2010; Lynch & Marinov, 2015) but also in the higher degree of synthesis of the EPSPS enzyme products, the reported lack of a fitness cost is surprising (especially for the massive EPSPS amplification observed in *A. palmeri*). This contradicts the theory described earlier on the biochemical origin of plant fitness costs. The results are even more interesting if we consider, in addition, that gene amplification occurring through gene insertions throughout the whole genome may potentially disrupt the expression and function of other genes and/or co-overexpress other genes in the replicon (although no other genes in the shikimate pathway have been found to be co-overexpressed in *K. scoparia* (Wiersma *et al.*, 2015).

Metabolic costs incurred in *EPSPS* gene amplification may be compensated for, provided that the energy invested in large amounts of protein and extra synthesis of Phe, Trp and Tyr Phe are recovered by amino acid catabolism. The concentration of free amino acids in cells can be regulated by a combination of transcriptional and post-translational control, allowing greater synthesis of amino acid catabolic enzymes if amino acid concentrations become too high (Hildebrandt *et al.*, 2015). Of all the amino acids, catabolism of Tyr has been shown to return the highest energy in ATP currency in plants (Hildebrandt *et al.*, 2015). A working hypothesis is that the energy cost invested in the massive EPSPS amplification of glyphosate-resistant *A. palmeri* might be compensated for by catabolism of the excess amino acids, particularly Tyr, produced by the amplified EPSPS activity.

Although it is reasonable to expect that the process of natural selection could have minimized the costs associated with *EPSPS* gene amplification (Andersson, 2003; Paris *et al.*, 2008; Darmency *et al.*, 2015), studies conducted over a single plant generation may not detect subtle fitness differences (Giacomini *et al.*, 2014; Vila-Aiub *et al.*, 2014; Kumar & Jha, 2015; Martin *et al.*, 2017) which only manifest themselves after several generations of additive fitness cost effects (each of which could also slowly reduce the frequency of plants carrying the amplified gene) (Vila-Aiub *et al.*, 2009b, 2011, 2015). For instance, the frequency of *A. tuberculatus* plants with up to five *EPSPS* gene copies grown in competition decreased from 50% to less than 5% after six generations without glyphosate treatment (Wu *et al.*, 2018). Multigenerational studies rely on the fact that a costly resistance allele will decrease in frequency over time, so any significant deviations from expected resistance

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genotypic frequencies provide clear evidence of the expression and magnitude of a fitness cost (Roux *et al.*, 2004).

Another study identified that EPSPS copy number in glyphosate-resistant *A. tuberculatus* was linearly correlated with higher glyphosate resistance and reproductive growth penalty compared with plants with no *EPSPS* gene amplification (Cockerton, 2013). The estimated 10% growth penalty at reproduction associated with EPSPS amplification was not expressed in plants grown without competition but was evident in plants under intraspecific competition, denoting an ecologically mediated mechanism. The magnitude of the reproductive growth penalty was surprisingly similar between plants carrying either 12 or 115 EPSPS copies, suggesting that the expected excess in energy cost in the latter was ameliorated (Cockerton, 2013). Interestingly, intense interspecific competition from maize moderated this detrimental fitness effect and thus no difference in seed production was evident between plants with and without EPSPS amplification, when they were grown with maize competition (Cockerton, 2013).

For a fitness cost mechanism in which extra energy and material investment is required to sustain a herbicide resistance level (but precluding their diversion to growth and reproduction), it has been predicted that the cost will be greatest when resources are limiting (Bergelson, 1994; Purrington, 2000). This hypothesis particularly applies to the massive *EPSPS* gene amplification observed in *A. palmeri* and thus it requires further research to elucidate whether fitness costs can be expressed under naturally 'more stressful' conditions requiring a higher energy budget, for example in producing chemical defences against herbivory over several generations.

If the lack of fitness costs associated with gene amplification/overexpression contradicts the expected metabolic cost (see Costs imposed by increased energy requirements for gene amplification/overexpression), an increase in plant fitness due to protein overproduction would probably demand a reformulation of theoretical paradigms, as suggested by recent reports on the fitness effects of a glyphosate resistance EPSPS rice transgene introgressed into weedy (*Oryza sativa f. spontanea*) and wild rice (*Oryza rufipogon*) (Lu *et al.*, 2014a; Wang *et al.*, 2014; Yang *et al.*, 2017).

A modified native *EPSPS* gene (EP3) from rice, under the control of the maize ubiquitin promoter, was introgressed into various weedy rice accessions. Transgenic F_2 crop-weed plants exhibited glyphosate resistance due to a two-fold higher EPSPS expression and 5–25% more EPSPS protein (Lu *et al.*, 2014a; Wang *et al.*, 2014). As expected, a significant increase (20–100%) in free cellular Trp concentrations was observed in transgenic compared with nontransgenic F_3 plants (Wang *et al.*, 2014). However, crop-weed plants overexpressing the *EPSPS* gene exhibited a remarkable increase in photosynthetic rate and fecundity. A similar fitness increase was also estimated in wild rice plants when transformed with the same EPSPS overexpression event (Yang *et al.*, 2017).

Again, overexpression of both *Agrobacterium* and *A. thaliana EPSPS* genes via the CaMV35S promoter resulted in about 35% higher EPSPS content in transgenic *A. thaliana*, endowing high-level glyphosate resistance and a 30% increase in silique and seed number per plant in glyphosate-free (controlled) environments (Fang *et al.*, 2018). This fitness benefit has been shown to correlate with a higher auxin content, which is probably derived from the extra synthesis of the amino acid Trp (Mashiguchi *et al.*, 2011; Fang *et al.*, 2018). In another study overexpressing a native *EPSPS* gene (again using the CaMV35S promoter) in several transgenic *A. thaliana* lines, there were no growth penalties, but a growth benefit was found only in a few transgenic events (Beres *et al.*, 2018).

It has been claimed that the expected higher metabolic cost associated with EPSPS overexpression may be offset if the concomitant higher concentration of EPSPS, its downstream products (aromatic amino acids, secondary compounds, lignin, auxin) and transcriptional regulatory functions (Xie *et al.*, 2018) endows a selective advantage in a glyphosate-free environment (Beres *et al.*, 2018; Fang *et al.*, 2018). In natural environments, it is possible that pressure from herbivory could select for glyphosate-resistant plants overproducing alkaloids and tannins via *EPSPS* gene amplification/overexpression. Thus, the estimation of associated fitness costs would require that experiments take place in natural field conditions, including insects and pests.

The reported fitness benefits in segregating transgenic plants overexpressing the *EPSPS* gene may result from fitness effects of the random positional insertions of the transgene disrupting other gene functions and metabolism, and/or linking with unrelated fitness genes. Nonetheless, these findings merit further research on the effects of this strongly expressed EPSPS transgene on plant metabolomics and ecology, which could provide insights into the mechanism by which increases in mRNA and EPSPS protein content and amino acid synthesis do not translate into energy costs limiting its evolution.

VIII. Evolutionary rescue of fitness costs

Through successive generations, the impact of a herbicide resistance allele/s on plant fitness can be modified by wider changes in the genome. Such compensatory evolution to reduce the adverse impact of a resistance mutation is known in the microbial and insecticide resistance literature (Guillemaud *et al.*, 1998; Björkman *et al.*, 2000) but there have been limited studies conducted with herbicide-resistant plants (Darmency *et al.*, 2015). Despite the high glyphosate resistance fitness cost associated with the TIPS mutation in *E. indica* (Han *et al.*, 2017), it is interesting to note that genetically modified glyphosate-resistant maize containing the TIPS mutation has no detectable fitness penalty (Spencer *et al.*, 2000). This genetic transformation event (GA21), however, included a strong promoter with three TIPS *EPSPS* gene copies in tandem (Monsanto, 2002). Indeed, it has been shown that the fitness cost observed in transgenic, glyphosate-tolerant cassava (*Manihot esculenta*) plants with the double EPSPS mutation can be compensated for by EPSPS overexpression via a strong CRISPR/Cas9-edited promotor (Hummel *et al.*, 2018). This means of rescuing plants with a fitness cost will remain as a working hypothesis, however, until EPSPS double mutations and overexpression are identified in single, naturally evolved plants displaying no fitness cost. Interestingly, the reduced PEP affinity associated with the TIPS mutation in *E. indica* (Yu *et al.*, 2015) could be a real limit for evolution in glyphosate-free environments (Han *et al.*,

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2017), as introduction of additional EPSPS mutations to compensate for the reduction in EPSPS catalytic efficiency has been unsuccessful (Dong *et al.*, 2019). However, multiple compensatory EPSPS mutations may provide a successful evolutionary pathway in plants for high-level resistance with fitter EPSPS, as already demonstrated in the laboratory (Weinreich *et al.*, 2006; Dong *et al.*, 2019). In fact, a triple EPSPS mutation (TIPS + Ala-103-Val) has recently been reported in glyphosate-resistant *A. hybridus* with high frequency (nearly 50% of the resistant individuals are homozygous for the triple mutation) (Perotti *et al.*, 2018), although no data on EPSPS activity or fitness cost are available.

As mentioned, where a resistance allele incurs a substantial fitness cost it is likely that natural selection of genetic elements at loci other than resistance genes will lead to evolution of fitness cost compensation (Bergelson & Purrington, 1996; Menchari *et al.*, 2008; Paris *et al.*, 2008; Vila-Aiub *et al.*, 2009b; Yu *et al.*, 2010; Darmency *et al.*, 2015). When considering the amplification of the *EPSPS* gene throughout the *A. palmeri* genome, it is important to note that it comprises not only the EPSPS locus (10 kb) but also genomic sequences corresponding to 71 putative genes, tandem repeats and regulatory elements (Gaines *et al.*, 2013; Molin *et al.*, 2017a). This *EPSPS cassette*, with a size of *c.* 300 kb, causes a genome size increase of about 10% in glyphosate-resistant plants with 100 *EPSPS* gene copies (Molin *et al.*, 2017a). The few polymorphisms in the flanking sequences to the EPSPS locus suggest that the *EPSPS cassette* has been subjected to little or no recombination and is probably the result of a selection sweep that led to its fixation in many glyphosate-resistant *A. palmeri* populations (Gaines *et al.*, 2013; Molin *et al.*, 2017a,b). In support of this speculation is the fact that the amplified *EPSPS cassette* includes genes linked to environmental stress (e.g. heat shock cognate 70 protein) which may provide an extra adaptive value to *EPSPS* amplification other than merely glyphosate resistance. If this were the case, other environmental factors would be selecting for *EPSPS* amplification despite any associated extra energy investment leading to fitness penalties.

IX. Final remarks

There are a number of factors at the molecular and physiological levels that lead to the expression of a plant fitness cost based on a tradeoff between EPSPS glyphosate resistance and EPSPS catalytic functionality. By artificial or natural evolution, several single and double target-site EPSPS mutations have been shown to code for a glyphosate-resistant EPSPS protein. Given that inhibition of EPSPS by glyphosate is competitive in relation to PEP, mutations that give structural changes in the EPSPS active site preventing efficient binding of both glyphosate and PEP will endow the highest glyphosate resistance with a concomitantly reduced EPSPS catalytic activity and plant fitness cost. This highlights both the importance of identification of the particular *EPSPS* resistance target-site mutation and the contribution of structural modelling and enzyme kinetic approaches in examining the molecular interactions between EPSPS variants, glyphosate and PEP binding, and the intrinsic fitness of the variants. Altogether, this knowledge can provide useful information for the prediction of fitness costs associated with glyphosate resistance in field-evolved weedy species and novel transgenic crops.

Based on the evolution of gene expression and resource allocation theory (reviewed in Herms & Mattson, 1992; Bergelson & Purrington, 1996; Lynch & Marinov, 2015), *EPSPS* gene amplification or overexpression should attract plant fitness penalties due to a metabolic cost. Some empirical evidence has validated this hypothesis, showing that evolved overproduction of EPSPS and downstream products incurs a fitness cost (Cockerton, 2013; Yanniccari *et al.*, 2016; Martin *et al.*, 2017; Wu *et al.*, 2018). However, the basis of this constrained energy budget under EPSPS amplification has been challenged, not only by those cases in which a cost has not been detectable (Giacomini *et al.*, 2014; Vila-Aiub *et al.*, 2014; Kumar & Jha, 2015; Martin *et al.*, 2017; Osipitan & Dille, 2019), but also in those studies reporting on a fitness advantage endowed by *EPSPS* overexpression in transgenic plants (Lu *et al.*, 2014a,b; Wang *et al.*, 2014; Yang *et al.*, 2017; Beres *et al.*, 2018; Fang *et al.*, 2018). It is possible that the expression of a fitness cost due to gene amplification might not be visible until the requirement for extra energy reaches a critical threshold. Alternatively, the associated cost might be moderate and only perceived after several generations through which fitness costs may be stacked. However, these hypotheses would not fit the massive *EPSPS* amplification present in *A. palmeri*, in which fitness of resistant plants is similar to plants without such EPSPS amplification. To explain the lack of expression of fitness costs in glyphosate-resistant *A. palmeri*, an estimation of the energy budget involved and elucidation of the role of the genes flanking *EPSPS* in the amplified *EPSPS cassette* will be helpful.

Although the introgression of resistance alleles into a susceptible background is a robust protocol for the detection of fitness costs (Vila-Aiub *et al.*, 2011), reports on fitness benefits from *EPSPS* overexpression in transgenic events need to be further validated until it can be confirmed that this remarkable finding is solely due to the glyphosate resistance transgene and its active promoter (Lu *et al.*, 2014a,b; Wang *et al.*, 2014; Yang *et al.*, 2017; Beres *et al.*, 2018; Fang *et al.*, 2018).

Understanding the underlying effects of glyphosate resistance alleles and mechanisms on plant molecular biology, biochemistry and physiology is pivotal for predicting the effects on plant fitness. Thus, for target-site EPSPS resistance to glyphosate, do plants pay a fitness cost? We conclude that naturally evolved target-site EPSPS mutations endowing high glyphosate resistance are more likely to reduce EPSPS catalytic activity and consequently endow a substantial plant fitness cost. Greater insights into the metabolic profile/consequence of *EPSPS* amplification and overexpression are required so that the prediction of associated fitness costs can become as accurate as those for target-site *EPSPS* gene mutations.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 EPSPS kinetics associated with glyphosate resistance target site EPSPS mutations.

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