Tansley insight

Molecular mechanisms of adaptive evolution revealed by global selection for glyphosate resistance

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Summary

The human-directed, global selection for glyphosate resistance in weeds has revealed a fascinating diversity of evolved resistance mechanisms, including herbicide sequestration in the vacuole, a rapid cell death response, nucleotide polymorphisms in the herbicide target (5-enolpyruvylshikimate-3-phosphate synthase, *EPSPS*) and increased gene copy number of *EPSPS*. For this latter mechanism, two distinct molecular genetic mechanisms have been observed, a tandem duplication mechanism and a large extrachromosomal circular DNA (eccDNA) that is tethered to the chromosomes and passed to gametes at meiosis. These divergent mechanisms have a range of consequences for the spread, fitness, and inheritance of resistance traits, and, particularly in the case of the eccDNA, demonstrate how evolved herbicide resistance can generate new insights into plant adaptation to contemporary environmental stress.

I. Introduction

The herbicide glyphosate, introduced in the mid-1970s, inhibits 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), a critical gene in aromatic amino acid synthesis. It is commonly used for nonselective weed control in many agricultural and nonagricultural settings, including transgenic glyphosate resistant crops. Since the mid-1990s, an increasing reliance on glyphosate has resulted in considerable selection for glyphosate resistance (Heap & Duke, 2018). Currently, 42 weed species have evolved glyphosate resistance across six continents.

Early evidence and speculation suggested that evolution of glyphosate resistance in weeds would be extremely rare. But the broad adoption of transgenic glyphosate-resistant crops and increases in glyphosate use have resulted in an unprecedented selection experiment that has revealed a diverse array of routes to resistance, including novel molecular genetic mechanisms. Here, we review those mechanisms, their inheritance and phenotypic consequences and consider the remaining unanswered questions

that address important fundamental and applied questions in plant adaptation.

II. Glyphosate resistance mechanisms

Herbicide resistance mechanisms can be classified as either target-site (mutations affecting herbicide inhibition of target-site proteins) or nontarget-site mechanisms (any mechanism that reduces the quantity and rate of herbicide accumulation at the target site). Glyphosate selection has revealed a wider range of molecular mechanisms of resistance (Fig. 1) than for any other herbicide mode of action (reviewed by Sammons & Gaines, 2014).

Target-site mutations in *EPSPS* have been documented at amino acid position Pro106 (reviewed by Sammons & Gaines, 2014), at Thr102 (Li *et al.*, 2018), at Thr102Ile and Pro106Ser in combination (the TIPS mutation, Yu *et al.*, 2015), and recently a triple mutation involving Thr102Ile, Ala103Val, and Pro106Ser (Perotti *et al.*, 2019). The simultaneous occurrence of multiple mutations within the same allele appears to be unique to glyphosate resistance and *EPSPS*. The TIPS and TIAVPS mutations confer much higher resistance to glyphosate than single mutations at Pro106, although it is not certain if the Ala103Val mutation adds additional resistance or affects EPSPS enzyme kinetics in the absence of glyphosate.

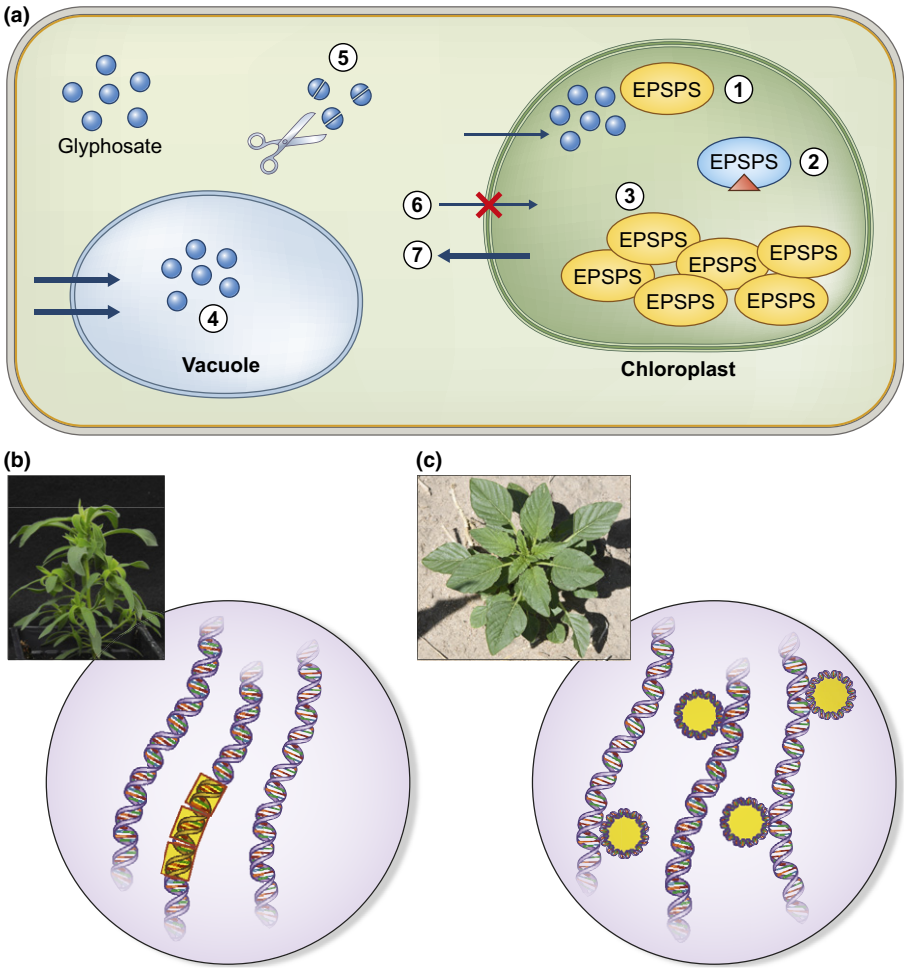

Fig. 1 (a) Observed and predicted glyphosate resistance mechanisms. In sensitive plants, glyphosate (blue circles) enters the cytoplasm and is transported into the chloroplast to the target site enzyme, *EPSPS* (1). Mutations changing one or two amino acids in *EPSPS* can confer target-site resistance, first reported in 2002 (2). Extra gene copies of *EPSPS* can produce extrasensitive EPSPS, requiring proportionally more glyphosate to cause complete inhibition, first reported in 2010 (3). Glyphosate can be transported and sequestered in the vacuole, with the mechanism first reported in 2010 and the observation of reduced glyphosate translocation first reported in 1999 (4). Some evidence to suggest glyphosate metabolism has been reported, but no specific genes have been identified in weeds to date (5). We predict that mechanisms might exist to reduce glyphosate import into the chloroplast (6) and/or rapidly export glyphosate out of the chloroplast (7), but neither mechanism has been observed to date. (b) *EPSPS* gene duplication in *Kochia scoparia* occurs as a 45–70 kbp tandem duplication at a single locus, with predictable inheritance and potential for changes in copy number in progeny as a result of unequal recombination; the duplication may have been triggered by insertion of a mobile genetic element next to *EPSPS*. (c) *EPSPS* gene duplication in *Amaranthus palmeri* occurs as a 300 kbp extrachromosomal circular DNA (eccDNA) carrying a single copy of *EPSPS*; the eccDNA is tethered via a protein to the chromatin and inherited biparentally, but the inheritance can show transgressive segregation of *EPSPS* copy number as a result of instability of the eccDNA. The origin of the eccDNA is unknown, and copy number of eccDNA can vary somatically from cell to cell.

Thus far, glyphosate resistance in weeds due to enhanced capacity to metabolize glyphosate has not been demonstrated. Enhanced glyphosate metabolism has been reported (e.g. Carvalho *et al.*, 2012) but further characterization is needed. Intriguingly, an overexpressed aldo-keto reductase gene was shown to confer a level of glyphosate resistance in rice, although no evidence of glyphosate metabolites from this transgene was provided (Vemanna *et al.*, 2017). Several candidate differentially regulated transcripts were identified using RNA-Seq in *Ipomoea purpurea*, including a cytochrome P450 gene (Leslie & Baucom, 2014). Further research into enhanced glyphosate metabolism in glyphosate resistant weeds is justified.

Reduced glyphosate translocation has been attributed to a vacuolar sequestration mechanism in *Conyza canadensis* (Ge *et al.*, 2011) and *Lolium* spp. (Ge *et al.*, 2012), but specific causative genes for reduced translocation have not yet been identified. ATP-binding cassette (ABC) genes have been associated with reduced translocation in *C. canadensis* but not yet functionally validated (Yuan *et al.*, 2010; Tani *et al.*, 2015).

An especially unique glyphosate resistance mechanism is the rapid cell death phenotype observed in *Ambrosia trifida* populations, in which all known candidate resistance mechanisms were absent and reactive oxygen species accumulated within 30 min of glyphosate application in older leaves of resistant individuals. (Moretti *et al.*, 2018; Van Horn *et al.*, 2018). The cause of this rapid response remains unknown, but the phenotypic similarities to effector-triggered immunity raise the intriguing possibility that a disease response pathway may have evolved a new function in herbicide resistance.

III. Gene duplication in multiple species through different molecular mechanisms

Increased *EPSPS* expression as a result of extra *EPSPS* gene copies has now been reported in eight species (reviewed by Patterson *et al.*, 2018). Recently, access to genomic resources, combined with cytogenetics, has provided critical evidence for our understanding of the molecular mechanisms of gene duplication (Jugulam & Gill, 2018).

Amaranthus palmeri: eccDNA

Amaranthus palmeri (Palmer amaranth) is the first species in which *EPSPS* gene duplication was reported, with resistant individuals carrying a variable number of extra *EPSPS* gene copies, ranging from 20 to > 100 (Gaines *et al.*, 2010). Original reports showing *EPSPS* in association with every chromosome attributed this to transduplication and introgression into chromosomes, a conclusion now proven to be incorrect (see next paragraph). Mobile genetic elements were identified within 1–2 kbp of sequence flanking the *EPSPS* gene in resistant but not susceptible individuals (Gaines *et al.*, 2013). Subsequently, an *c.* 300 kbp sequence containing *EPSPS* was identified using bacterial artificial chromosome (BAC) sequencing (Molin *et al.*, 2017a) with substantial sequence differences between resistant and susceptible individuals upstream and downstream of the *EPSPS* gene. The replicon containing *EPSPS* contained 71 additional predicted open reading frames, including genes with transposase domains, heat shock proteins, a reverse transcriptase gene, various categories of repetitive sequences, and, perhaps most significantly, sequences with homology to autonomous origins of replication (Molin *et al.*, 2017a).

In a recent, major discovery, Koo *et al.* (2018) identified an extrachromosomal circular DNA (eccDNA) carrying *EPSPS* in *A. palmeri* mitotic cells. The eccDNA was inherited at meiosis but not in equal proportions among gametes (Fig. 1) and was observed to be tethered to chromosomes by a structural protein, which enables transmission from cell to cell during mitosis and meiosis, a first for eukaryotic cells, although previously reported for some autonomously replicating viruses (reviewed in Koo *et al.*, 2018). The *A. palmeri* eccDNA is much larger at 300 kbp than previously reported in plants (2–20 kbp; Cohen *et al.*, 2008).

Kochia scoparia: tandem duplication

Kochia scoparia populations were identified with three to 10 extra *EPSPS* gene copies (Wiersma *et al.*, 2015). Cytogenetics experiments revealed that the extra *EPSPS* gene copies were arranged as a tandem duplication at a single locus (Fig. 1) (Jugulam *et al.*, 2014). A positive correlation between degree of resistance and number of *EPSPS* copies has been established (Gaines *et al.*, 2016). Sequencing inserts from a *K. scoparia* genomic BAC library identified seven coduplicated genes and two different sizes of duplicated sections (Patterson *et al.*, 2019). A key observation is the insertion of additional sequence containing mobile genetic elements such as a Far1 transposon next to the *EPSPS* gene in glyphosate-resistant *K. scoparia* that is absent in glyphosate-susceptible individuals. The model proposed is that this repetitive sequence insertion generated a site for unequal recombination, leading to the generation of extra *EPSPS* gene copies that had a selective advantage under glyphosate selection (Patterson *et al.*, 2019).

Other species: unknown molecular mechanisms

Descriptions of *EPSPS* gene duplication have been reported for four grass weed species (reviewed in Patterson *et al.*, 2018), but less is known about the molecular mechanism(s) by which this gene duplication has occurred in grasses than in the dicotyledonous species *K. scoparia* and *A. palmeri*. In *Amaranthus tuberculatus* (waterhemp), glyphosate-resistant individuals had from four to eight extra *EPSPS* gene copies (Lorentz *et al.*, 2014). Cytogenetics experiments revealed tandem *EPSPS* gene duplication followed by seemingly rare excision of a small extra chromosome resulting in higher *EPSPS* gene copy number (Dillon *et al.*, 2017).

IV. Evolutionary dynamics of a 'rare' resistance trait

Twenty years of escalating glyphosate use has facilitated an unprecedented, human-directed selection experiment. Speculation that glyphosate might be uniquely robust to resistance evolution has been disproved, and globally distributed weedy plant species have exhibited convergent evolution of glyphosate resistance, with

four generalized 'types' of resistance confirmed: an altered *EPSPS* sequence; altered patterns of glyphosate sequestration and translocation; rapid cell death; and *EPSPS* gene duplication. As novel molecular mechanisms have emerged, experimental and theoretical studies have addressed the origins, spread, fitness consequences, and inheritance of glyphosate resistance. Unsurprisingly, given the diversity of species, molecular mechanisms, and selection regimes, these studies identify that the evolutionary, quantitative, and population genetics underpinning glyphosate resistance mechanisms are equally diverse.

The rapid evolution and spread of resistance from the late 1990s onwards, 20 yr after its first use, raises questions about the origin and spread of resistance mutations. Did escalating selection after the mid-1990s lead to the spread of rare resistance mutations that arose as single evolutionary events or were there multiple, independent evolutionary events within a species and/or species complex? Based on the size and sequence of the amplified *EPSPS* cassette in geographically distinct *A. palmeri* populations, Molin *et al.* (2017b) concluded that resistance probably arose as a single evolutionary event. Using a genotyping-by-sequencing approach in the same species, Küpper *et al.* (2018) found distinct population genetic structure between glyphosate-resistant populations from Georgia and Tennessee, suggesting two or more origins of glyphosate resistance may be possible. Similarly, Okada *et al.* (2013) demonstrated multiple origins of glyphosate resistance in populations of *Conyza canadensis* in the Central Valley of California. Recently, multiple origins of glyphosate resistance were identified in *A. tuberculatus* by whole-genome resequencing, showing both the introduction of glyphosate resistance into Canada from the USA as well as independent evolution of glyphosate resistance in other populations in Canada (Kreiner *et al.*, 2018). Others have shown that glyphosate resistance traits can be spread through hybridization between related *Amaranthus* species (Gaines *et al.*, 2012; Nandula *et al.*, 2014). The recent appearance of glyphosate-resistant populations of *A. palmeri* in Brazil with *EPSPS* gene duplication hints at the potential for intercontinental spread of resistance mutations, mediated by seed movement (Küpper *et al.*, 2017). Experiments have demonstrated that glyphosate-sensitive populations of *L. rigidum* (Busi & Powles, 2009) and *Alopecurus myosuroides* (Davies & Neve, 2017) harbor heritable standing variation for glyphosate insensitivity, although this additive genetic variation may be distinct from the mechanisms of resistance selected in field-evolved resistant weed populations. Selection for glyphosate resistance and susceptibility in diverse lines of *Ipomoea purpurea* also demonstrated heritable standing genetic variation for glyphosate insensitivity (Debban *et al.*, 2015).

Recent resequencing of the eccDNA from divergent glyphosate-resistant *A. palmeri* populations has shown that the eccDNA is nearly identical in every glyphosate-resistant individual, with no structural variation and very few single nucleotide polymorphisms (SNPs; Molin & Saski, 2019). It would be highly unlikely for two individuals to independently generate eccDNA that were so nearly identical. As the eccDNA is not integrated with the genome, it is in full linkage disequilibrium with the entire nuclear genome; therefore, there is no linkage drag associated with glyphosate resistance inheritance via eccDNA. This allows for any nonadaptive loci from an introduced resistant genome to be purged quickly from the local population via backcrossing (especially as *A. palmeri* is dioecious and almost exclusively outcrossing). This may be why the nuclear genomes from two distant *A. palmeri* populations are divergent, as described earlier (Küpper *et al.*, 2018), but the *EPSPS* eccDNA is nearly identical (Molin & Saski, 2019).

The fitness costs and benefits of glyphosate resistance mechanisms vary according to species and mechanism, with mechanisms based on the TIPS mutation and eccDNA-based duplication providing high amounts of resistance, while other mechanisms provide moderate to low resistance. It has been speculated that the relatively slow evolution of glyphosate resistance may reflect high fitness costs associated with resistance mutations. However, there is little evidence for universally high costs, except for the TIPS mutation (reviewed in Vila-Aiub *et al.*, 2019).

For most known glyphosate resistance mechanisms, inheritance studies have established segregation of resistance traits as single dominant or semidominant loci (reviewed in Powles & Preston, 2006). Indeed, even the gene duplication mechanism in *K. scoparia* appears to segregate as a single locus, albeit the actual gene copy number may vary from generation to generation through unequal crossing over (Jugulam *et al.*, 2014). The recent report of a glyphosate resistance mechanism mediated by eccDNA gene duplication begins to shed light on the transgressive segregation of copy number variation observed for *A. palmeri* populations possessing this mechanism (Gaines *et al.*, 2011), highly variable within plant copy number, and increased *EPSPS* copy number in progeny of single copy parental plants, suggesting that a few cells may retain eccDNA and transmit them to progeny (Giacomini *et al.*, 2019). The eccDNA-based mechanism and potentially other novel mechanisms such as mobile genetic element insertion driving gene duplication are suggestive of a greater role for mechanisms that generate novel genetic variation in somatic cells (sporophyte genomes), which is subsequently transmitted into gametes via differentiation of somatic cells into germline cells during plant development. Many questions relating to the origins, dispersal, inheritance, and fitness consequences of this fascinating mechanism of resistance call into question the paradigms and models that have informed our current understanding of the evolutionary dynamics of herbicide resistance.

V. Conclusions

Under intense glyphosate selection, different species have converged on a range of molecular solutions to evolve resistance. For some of these mechanisms, for example, target site resistance based on nucleotide polymorphisms, conventional Darwinian models of random mutation and subsequent selection suffice to explain observed patterns of adaptation. It is, however, increasingly evident that other evolutionary mechanisms may be responsible for generating the genetic variation on which selection can act. This genetic variation may arise from epigenetic modification, genomic rearrangements, gene copy number variation (CNV) and other mechanisms of genomic plasticity, and this variation is often inherited in a nonMendelian fashion.

The heritable, chromosome-tethered eccDNA in *A. palmeri* is the first such example in plants. The formation of eccDNAs is relatively common in plants (Cohen *et al.*, 2008; Lanciano *et al.*, 2017), including retrotransposons captured outside the chromatin in eccDNA form (Lanciano *et al.*, 2017), but, importantly, these eccDNAs are usually transient and not stably inherited. This raises a number of critical questions. How did the tethering mechanism that enables transmission of the eccDNA arise? Is contemporary adaptation based on eccDNA-based mechanisms a quirk of glyphosate selection, through which *A. palmeri* has co-opted a mechanism for genome housekeeping for adapting to a uniquely novel selection pressure? Another proposed role for eccDNA is to counteract genome expansion following interspecific hybridization (Cohen *et al.*, 2008). Could the eccDNA in *A. palmeri* be a by-product of the common interspecific hybridization in the genus *Amaranthus*? Or is eccDNA a more general mechanism for generating heritable genetic variation, and, if yes, is variation generated at random or is the mechanism stress-responsive?

The discovery of eccDNA-based mechanisms of resistance highlight the evolution of glyphosate (and herbicide) resistance as fertile ground to address fundamental and applied questions relating to plant adaptation (Baucom, 2019). In relation to the origins of resistance-conferring mutations, a key question that remains to be answered is: 'Does herbicide-induced stress result in CNV and genomic rearrangements?' Even if this were shown to be the case, it is intriguing to speculate how stochastic and infrequent cellular events, induced by exposure of a multicellular organism to a toxophore, could result in the generation of sufficient somatic variation to confer a resistant phenotype at the whole organism level. Beyond this, given the observation by Koo *et al.* (2018) of highly variable patterns of mitotic and meiotic transmission on eccDNA-based CNV, and of apparent tissue-to-tissue variation in eccDNA, several questions remain regarding how this variation influences the expression of the whole-plant resistance phenotype. The exciting discovery of this resistance mechanism adds fresh impetus to further population genetics/genomics-based studies to determine if the continent-wide evolution of resistance in *Amaranthus* species in North America (and beyond, potentially into South America) is based on a single, a few, or multiple evolutionary events. These studies will, in turn, inform the generality of eccDNA as a potential mechanism for resistance evolution.

The global, ongoing and human-directed selection for glyphosate resistance in weedy plants provides a lens through which to view, explore, and understand the range of mechanisms that underpin contemporary plant adaptation to environmental stresses. The inherent instability of these mechanisms is anticipated to affect the ecoevolutionary dynamics of glyphosate resistance in fundamentally different ways from other resistance mechanisms mediated by SNPs. The diversity of glyphosate resistance mechanisms requires evaluation using different evolutionary models that consider inheritance, fitness penalty, genome dynamics and plant physiology. Research on glyphosate resistance has already proved to be a valuable field for challenging and expanding classic adaptive evolution paradigms and it will continue to do so in the future.

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