Glyphosate resistance and *EPSPS* gene duplication: Convergent evolution in multiple plant species

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Running title: *EPSPS* gene duplication

ABSTRACT

One of the increasingly widespread mechanisms of resistance to the herbicide glyphosate is copy number variation (CNV) of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene. *EPSPS* gene duplication has been reported in eight weed species, ranging from 3-5 extra copies to more than 150 extra copies. In the case of Palmer amaranth (*Amaranthus palmeri*), a section of >300 kb containing *EPSPS* and many other genes has been replicated and inserted at new loci throughout the genome, resulting in significant increase in total genome size. The replicated sequence contains several classes of mobile genetic elements including helitrons, raising the intriguing possibility of extra-chromosomal replication of the *EPSPS*-containing sequence. In kochia (*Kochia scoparia*), from three to more than 10 extra *EPSPS* copies are arranged as a tandem gene duplication at one locus. In the remaining six weed species that exhibit *EPSPS* gene duplication, little is known about the underlying mechanisms of gene duplication or their entire sequence. There is mounting evidence that adaptive gene amplification is an important mode of evolution in the face of intense human-mediated selection pressure. The convergent evolution of CNVs for glyphosate resistance in weeds, through at least two different mechanisms, may be indicative of a more general importance for this mechanism of adaptation in plants. CNVs warrant further investigation across plant functional genomics for adaptation to biotic and abiotic stresses, particularly for adaptive evolution on rapid time scales.

Introduction

The herbicide glyphosate has been described as a "once-in-a century-herbicide" due to its unique broad spectrum of weed control efficacy (Duke and Powles 2008). It inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) which is found in both monocotyledon and dicotyledon plants (Steinrücken and Amrhein 1980). EPSPS catalyzes the reaction that metabolizes 3-phosphoshikimate into 5-enolpyruvylshikimate-3-phosphate, an essential step in the synthesis of aromatic amino acids. It is thought that glyphosate causes plant death by starving them of aromatic amino acids (Schönbrunn et al. 2001). The ecological toxicity profile of glyphosate has been shown to be extremely low due to rapid metabolism by soil microbes and tight binding of the chemical to soil (Giesy et al. 2000; Rueppel et al. 1977; Williams et al. 2000). Additionally, EPSPS is found only in plants and microorganisms with no homolog in animals (Herrmann and Weaver 1999). Glyphosate was introduced as a herbicide in the early 1970s (Baird et al. 1971) and has been used in non-selective applications (e.g., orchards, vineyards, fallow, prior to planting broadacre crops, postharvest) since its introduction. Beginning in 1996, the introduction of transgenic glyphosate-resistant crops including cotton, soybean, sugar beet, and corn extended glyphosate use to selective in-crop application (Duke and Powles 2008; Padgette et al. 1996).

The commercially successful transgenic glyphosate-resistant crops contain a gene of bacterial origin (*CP4 EPSPS*) that is glyphosate-insensitive and therefore confers a high level of resistance in plants (Padgette et al. 1996). However, attempts to discover genetic variation for glyphosate resistance in crops provide insights into the natural selection of glyphosate resistance in weeds. Several molecular and genetic approaches were utilized to develop glyphosate-resistant crops, although most of these were not commercialized. A perennial ryegrass variety was recurrently selected with increasing doses of glyphosate over 11 generations, but this selection experiment resulted in only moderate resistance (Johnston and Faulkner 1991). Chemical mutagenesis of over 1 million *Arabidopsis thaliana* seeds did

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not produce any resistant plants, leading to the conclusion at the time that a single point mutation in the target-site plant *EPSPS* may not be sufficient to confer resistance (Bradshaw et al. 1997; Haughn and Somerville 1987). Liquid plant cell cultures of chicory, petunia, tobacco, tomato, and carrot were exposed to increasing amounts of glyphosate and eventually some of the cells became resistant to the glyphosate in the media by over-expressing *EPSPS*, sometimes by increases in gene copy number (Goldsbrough et al. 1990; Nafziger et al. 1984; Sellin et al. 1992; Shyr et al. 1993; Smith et al. 1986; Steinrücken et al. 1986; Wang et al. 1991). These resistant cell lines typically had issues that prevented their commercial release, such as instability of the increase in *EPSPS* gene copy number upon regeneration to a whole plant, loss of glyphosate resistance on regeneration, or infertility of the regenerated plant following glyphosate application. Experiments in alfalfa, soybean, and tobacco further demonstrated that *EPSPS* gene amplification can confer glyphosate resistance in plants (Widholm et al. 2001). Ultimately, the recurrent selection, mutagenesis, and cell culture methods suggested that there is limited standing genetic variation for glyphosate resistance in plants.

The first case of a naturally evolved glyphosate-resistant (GR) weed was annual ryegrass (*Lolium rigidum*), discovered in Australia in an orchard (Powles et al. 1998). To date, 37 species have been reported as GR (Heap 2017). These 37 species include both monocotyledon and dicotyledon weeds. Glyphosate resistance has evolved in a variety of situations including orchards, cereals, fence lines, and transgenic GR crops. Glyphosate resistance in weeds can be conferred by several genetic mechanisms including point mutations in the active (target) site of *EPSPS*, reduced translocation of glyphosate to the meristems, and vacuole sequestration (reviewed by Sammons and Gaines 2014). One of the most interesting and increasingly widespread mechanisms of resistance to glyphosate is increased copy number of the *EPSPS* gene. In this review, we discuss the current information for each species that has evolved increased *EPSPS* gene copy number as a resistance mechanism and synthesize the current state of knowledge for this striking case of convergent evolution. We suggest that adaptive gene amplification can

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be an important mode of evolution on rapid time scales in the face of intense human-mediated selection pressure.

EPSPS Copy Number Variation

An increase in copy number of a gene produces copy number variation (CNV), referred to as gene amplification or gene duplication. *EPSPS* gene duplication is thought to confer resistance to glyphosate by over-production of the target protein, EPSPS. The increased protein pool of EPSPS requires an equivalent increase in applied glyphosate to inhibit sufficient amounts of EPSPS to cause lethality (Gaines et al. 2010; Sammons and Gaines 2014). Additionally, since glyphosate binding to the EPSPS protein is essentially irreversible, once glyphosate is bound it is effectively sequestered by the plant.

The first demonstration that *EPSPS* gene duplication confers glyphosate resistance was in Palmer amaranth (*Amaranthus palmeri*) from Georgia, USA (Gaines et al. 2010). Six additional weedy species have independently evolved increased *EPSPS* copy number and one species has obtained high *EPSPS* copy number by hybridization with GR Palmer amaranth (Chen et al. 2015; Lorentz et al. 2014; Malone et al. 2016; Nandula et al. 2014; Ngo et al. 2017; Salas et al. 2012; Wiersma et al. 2015). To date four of the resistant species are dicotyledons in the Chenopodiaceae/Amaranthaceae and four are monocotyledons in the Poaceae.

Palmer amaranth

GR Palmer amaranth was first reported in the US state of Georgia (Culpepper et al. 2006). Since that time, GR Palmer amaranth has become a substantial problem in several major crops in North and South America (Küpper et al. 2017; Norsworthy et al. 2014; Price et al. 2011; Sosnoskie and Culpepper 2014). Quantitative PCR using relative quantification with a single copy normalization gene has demonstrated that resistant Palmer amaranth contains from 50 to more than 150 copies of the *EPSPS* gene

(Gaines et al. 2011; Küpper et al. 2017). In this species, increased *EPSPS* gene copy number is directly proportional to *EPSPS* mRNA and EPSPS protein abundance which is proportional to the quantity of glyphosate needed to control these plants (Gaines et al. 2010).

Cytogenetics approaches have proven highly useful in characterizing the molecular structure of gene duplications involved in herbicide resistance (Jugulam and Gill 2017). Cytogenetic studies using Fluorescence In Situ Hybridization (FISH) in GR Palmer amaranth showed that the *EPSPS* copies are dispersed across the genome on all chromosomes (Gaines et al. 2010). The duplicated *EPSPS* copies were shown to contain introns, indicating the duplication did not occur via an RNA-transposon, and multiple types of mobile genetic elements were found to be associated with the duplicated *EPSPS* genes (Gaines et al. 2013). More recently this has been confirmed using genomics (Molin et al. 2017a). The amplified region that contains *EPSPS* was sequenced by generating a BAC library and probing for the *EPSPS* gene and then sequencing those clones with long read Pacific Biosciences sequencing technology. The amplified region was found to be ~300 kb, in high abundance (>100 copies), and dispersed across the genome (Molin et al. 2017a). Flow cytometry measurements for GR Palmer amaranth individuals show significantly larger genomes than glyphosate-susceptible (GS) Palmer amaranth due to the large size and high copy number of the *EPSPS* replicon. Calculations show the GR genome to be between 20-30 Mbp (7-13%) larger than the GS genome (Molin et al. 2017a).

The amplified region contains 72 predicted genes, many of which were classified as transposable elements (TEs) based on a repetitive element database (Jurka et al. 2005), including LTR retrotransposons, non-LTR retrotransposons, class II transposons, and helitrons (Molin et al. 2017a). Several of the genes in this region show increased transcription but not always to the same magnitude as *EPSPS* suggesting that either 1) not all genes in the amplified region are always duplicated or 2) these other genes are regulated differently than *EPSPS* (Molin et al. 2017a). The potential that the >300 kb replicon may have a circular structure is especially intriguing, inviting speculation that the entire structure

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could replicate externally to the chromosome and insert and excise repeatedly throughout the genome. This is the first documented case of such a potentially mobile, large genetic structure associated with gene duplication and copy number variation in any species.

To understand inheritance of the resistance trait, several studies with GR Palmer amaranth crossed to susceptible plants measured *EPSPS* copy number in the F1 and F2 progeny (Chandi et al. 2012; Mohseni-Moghadam et al. 2013). As would be expected due to the large number of *EPSPS* gene copies and their distribution across multiple, unlinked locations on different chromosomes, inheritance of glyphosate resistance in these studies was non-Mendelian and segregated as a polygenic trait. There are also indications that Palmer amaranth can produce seeds asexually via facultative apomixis (Ribeiro et al. 2014), which may facilitate inheritance of the potentially meiotically-unstable *EPSPS* gene duplication when it occurs via transduplication throughout an individual plant genome. A segregating F_2 population contained individuals with complete loss of the *EPSPS* replicon (*EPSPS* copy number of one) as well as individuals with *EPSPS* gene copy number greater than the sum of both parents (Gaines et al. 2011). The apparent instability of the *EPSPS* CNV raises questions about the likelihood of multiple independent CNV events versus a single origin and spread, as spread via gene flow could be dependent on the stability of transmission of increased *EPSPS* gene copy number across multiple generations. Resequencing and alignment of the *EPSPS* replicon from multiple glyphosate-resistant populations across the USA showed high sequence homology, supporting a hypothesis of single origin of the *EPSPS* replicon in Palmer amaranth (Molin et al. 2017b). At this point in time, some combination of both multiple origins (convergent evolution) and spread via seed- and pollen-mediated gene flow seems most likely (Beard 2014).

Some mutations conferring herbicide resistance have associated fitness costs including reduced growth rate, fecundity, and/or competitiveness due to direct or pleiotropic effects of the mutation (reviewed by Vila- Aiub et al. 2009). The *EPSPS* gene duplication in Palmer amaranth could affect plant

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fitness (growth rate, fecundity, competitiveness) in several ways, including 1) the increased metabolic cost of *EPSPS* overproduction; 2) potential pleiotropic effects of over-expressing other genes in the replicon; and 3) genome instability and disruption of other genes due to *EPSPS* insertion events. Two separate studies found no observable fitness costs in physiological traits (Giacomini et al. 2014; Vila-Aiub et al. 2014). However, since Palmer amaranth is dioecious and therefore an obligate outcrossing species, no studies have used near isogenic lines for conclusive fitness studies. Indeed, due to the size, dispersion, and potential instability of the *EPSPS*-containing replicon, obtaining true-breeding lines may not be possible. There may also be other fitness related traits that have not yet been measured that may demonstrate fitness costs of *EPSPS* gene amplification and genome expansion in Palmer amaranth.

Other *Amaranthus* Species

After the initial discovery of *EPSPS* gene amplification in Palmer amaranth, other GR *Amaranthus* weeds were evaluated for this mechanism. *EPSPS* copy number increase was described in waterhemp (*A. tuberculatus* syn. *rudis*) in several independent studies (Chatham et al. 2015a; Chatham et al. 2015b; Lorentz et al. 2014). *EPSPS* copy number in waterhemp was far fewer than in Palmer amaranth, with most resistant plants having between 4-8 copies up to a maximum of 16 copies (Chatham et al. 2015a; Chatham et al. 2015b). Dillon et al. (2017) grouped GR waterhemp into the following three categories of resistance magnitude: low glyphosate resistance (2-4 copies), moderate glyphosate resistance (4-7 copies), and high glyphosate resistance (7-16 copies). As shown in Palmer amaranth, genomic copy number was correlated with mRNA levels, shikimate accumulation (a biomarker for glyphosate inhibition of *EPSPS*), and glyphosate resistance level (Dillon et al. 2017). A fitness cost for increased *EPSPS* gene copy number in waterhemp was shown by a reduction in frequency of individuals carrying two or more *EPSPS* copies in a population grown for six generations without glyphosate selection (Wu et al. 2017).

Using FISH, it was discovered that the original copy of *EPSPS* in waterhemp is near the centromere in GS individuals (Dillon et al. 2017). There are several copies of *EPSPS* in tandem duplication at the same locus, near the centromere, in GR high copy number individuals. In the highest copy number individuals the *EPSPS* gene was also found on an extra chromosome, suggesting that tandem duplication may occur initially followed by transduplication and potentially replication of an extra chromosome (Dillon et al. 2017).

GR spiny amaranth (*Amaranthus spinosus*) exhibited up to a five-fold resistance to glyphosate in plants containing between 33-37 copies of *EPSPS* (Nandula et al. 2014). When the *EPSPS* gene was sequenced from GR individuals, the *EPSPS* gene was found to be identical to the gene from GR Palmer amaranth, having 29 single nucleotide polymorphisms when compared to the *EPSPS* gene from GS spiny amaranth. This evidence pointed to a hybridization event of spiny amaranth with high-copy number GR Palmer amaranth (Nandula et al. 2014). Inter-specific hybridization is known to occur within the *Amaranthus* genus (Trucco et al. 2005a; Trucco et al. 2005b; Trucco et al. 2009), including gene flow from Palmer amaranth to spiny amaranth (Gaines et al. 2012) and transfer of acetolactate synthase inhibitor resistance alleles between *Amaranthus* spp. (Franssen et al. 2001).

Kochia scoparia

Kochia scoparia (kochia) is a weed species in the Amaranthaceae common to the western Great Plains region of North America (Friesen et al. 2009) and GR kochia is a major agronomic challenge in this region (Kumar et al. 2014; Waite et al. 2013). The genus *Kochia* is related to the genus *Amaranthus* within the Amaranthaceae. Kochia has also evolved increased *EPSPS* copy number for glyphosate resistance (Godar et al. 2015; Wiersma et al. 2015), and currently is the only dicotyledon not in the *Amaranthus* genus with *EPSPS* CNV. Initially, GR kochia was shown to have *EPSPS* copy numbers between 3-9 (Kumar et al. 2015; Wiersma et al. 2015); however, in a survey from sugar beet fields, kochia plants were shown to occasionally have >10 copies of *EPSPS* (Gaines et al. 2016). Increased copy

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number has been correlated with increased mRNA and protein abundance as well as whole-plant resistance level in kochia (Gaines et al. 2016; Godar et al. 2015; Wiersma et al. 2015).

FISH in kochia has revealed that all copies of *EPSPS* occur at a single locus and Fiber-FISH suggests that all copies are located as a tandem duplication (Jugulam et al. 2014). Additionally, the Fiber-FISH results suggest several sizes for the tandem repeats, with the two most common being a repeat of ~45kb and a repeat of ~66kb. Additionally, some copies are slightly longer, >70kb, and one inversion was detected. The tandem duplication of *EPSPS* was proposed to be caused by an initial unequal crossing-over event that produced tandem *EPSPS* gene copies, followed by glyphosate selection pressure and further unequal crossing-over events during cell division that produced additional *EPSPS* copies in tandem duplication (Jugulam et al. 2014). Inheritance of the tandem *EPSPS* gene duplication was consistent with a single-gene pattern, as expected for a tandem duplication at a single locus (Jugulam et al. 2014).

An initial fitness study comparing high-copy number GR to GS kochia showed little to no fitness cost in most vegetative traits and little effect on reproductive traits (Kumar and Jha 2015). The two populations were collected from the same locality, but it is unknown how similar the genetic background is between the populations (Kumar and Jha 2015). More recently, researchers have made several crosses between GS and GR plants of varying copy number and measured several traits in the segregating F_2 population(s) (Martin et al. 2017). Some plants with elevated *EPSPS* copy number had delayed development, reduced fecundity, and reduced competitive ability. However, there was large variation among independent F_2 crosses in the magnitude of observed fitness costs, with fitness costs being either higher or absent depending on the specific cross (Martin et al. 2017). When comparing several GR and GS kochia populations in another study, it was observed that fitness costs were consistently found in germination characteristics but not necessarily in any vegetative characteristics (Osipitan and Dille 2017).

The Grasses

Several grass species in divergent genera of Poaceae appear to have independently evolved increased *EPSPS* copy number as a glyphosate resistance mechanism. Current information is limited to the occurrence of *EPSPS* gene duplication in the grasses, as no cytogenetic or sequencing studies have been completed. The species are Italian ryegrass (*Lolium perenne* ssp. *multiflorum*), ripgut brome (*Bromus diandrus*), goosegrass (*Eleusine indica*), and windmill grass (*Chloris truncata*), occurring in the USA, Australia, China, and Australia, respectively (Chen et al. 2015; Malone et al. 2016; Ngo et al. 2017; Salas et al. 2012). In all four grass species, increased copy number was associated with increased glyphosate resistance. In Italian ryegrass, *EPSPS* copy numbers were reported from 15 to 25 (Salas et al. 2012). In ripgut brome, *EPSPS* copy number ranged from 10 up to 36 copies (Malone et al. 2016). In goosegrass, *EPSPS* copy number was 89 in one population, 23-fold more copies than a susceptible population (Chen et al. 2015). Finally, in windmill grass, *EPSPS* copy number was reported from 32 up to 48 copies (Ngo et al. 2017). In these grass species, the inheritance, potential fitness costs, and cytogenetics of the *EPSPS* duplication events have not yet been reported.

Mechanisms of Copy Number Variation

Gene duplication is a relatively common process in evolutionary history and produces important raw material for adaptive evolution in mammalian cancer cells, bacteria, arthropods, and plants (Bass and Field 2011; Flagel and Wendel 2009; Gaines et al. 2010; Hastings et al. 2009; Schimke 1986; Wiersma et al. 2015). Plants can acquire additional gene copies in several ways. Mobile genetic elements such as transposable elements (TEs) are a well-studied mechanism of gene duplication. TE activity is usually suppressed because TE activity can have negative effects such as disrupting important genes or affecting

their transcription, or causing genome instability (Jensen et al. 1999; Slotkin and Martienssen 2007). There is some evidence, however, that certain biotic and abiotic stresses can increase TE activity, resulting in genomic re-arrangements (Bennetzen 2005; Capy et al. 2000). These rearrangements can be the duplication of genes contained within the TE boundaries, the movement of regulatory elements, the disruption of genes near the TE insertion site, or changes in chromatin structure (Bennetzen 2005).

The type of mobile genetic element recently identified in Palmer amaranth shares similarities with helitron structures (Molin et al. 2017a). Helitrons are a type of transposable element that are hypothesized to use a "rolling circle" replication mechanism, mediated by a single stranded DNA intermediate (Kapitonov and Jurka 2001; Kapitonov and Jurka 2007; Thomas and Pritham 2015). Helitrons were first discovered in Arabidopsis and rice but have since been discovered in almost all eukaryotic lineages. Helitrons can be quite prevalent in some eukaryotic genomes, ranging from 0-5% of the total genetic content. The helitron-like sequence that is associated with *EPSPS* gene duplication in Palmer amaranth alone can cause a >5% increase in genome size (Molin et al. 2017a).

Another possibility for generating increased gene copy number is tandem duplication events. For tandem duplications to occur, unequal crossing-over must occur between homologous chromosomes. In humans, tandem duplication events are known to be generated by one of two mechanisms: non-allelic homologous recombination (NAHR) and microhomology-mediated events (Hastings et al. 2009). Anytime a double stranded break (DSB) occurs in a strand of DNA, the subsequent repair to the damaged location may introduce mistakes, such as if the repair proteins accidentally employ NAHR or microhomology-based unequal recombination while the damage is being repaired (Hastings et al. 2009). These events can happen in somatic or gametic cells, but only events in gametes or somatic cells that eventually differentiate into gametes are heritable and therefore relevant to evolution. Because plant somatic cells are totipotent and can differentiate into gametic cells at various stages, especially in long-lived plants, a mechanism exists by which somatic variation can eventually be incorporated into gametes.

It is likely that a DSB or some other disruption near the *EPSPS* gene caused kochia to employ one of these unequal crossing-over mechanisms, inadvertently generating the tandem *EPSPS* duplications and copy number variation observed in this species (Jugulam et al. 2014).

Another way to generate additional copies of genes is via a polyploid event or gene flow from one organism to another. Polyploidy often shapes large-scale evolutionary events like speciation or genetic isolation and seems to be a relatively rare mechanism leading to single gene copy number changes, especially on short time scales (Adams and Wendel 2005; Ramsey and Schemske 1998). As previously mentioned, interspecific gene flow has occurred from Palmer amaranth to spiny amaranth, transferring duplicated copies of the *EPSPS* gene and glyphosate resistance (Gaines et al. 2012; Nandula et al. 2014).

In both animal and plant systems, it has been shown that environmental stress induces higher frequencies of CNVs (Hastings et al. 2000). The exact nature of the relationship between stress and CNVs is unclear. It could be that stress induces higher levels of DSB, resulting in more chances for gene duplications to occur and generate genetic diversity. Additionally, stress has been shown to change methylation patterns in several species which may be a way to regulate TE activity or the rate of DSB in certain genomic locations (Lämke and Bäurle 2017). There is evidence that unequal crossing-over events and TE insertions happen at hotspots mediated either by specific DNA sequences, epigenetics, or chromatin structure (Cai and Xu 2007; Drouaud et al. 2013; Gaut et al. 2007; Purandare and Patel 1997).

Copy Number Variation and Adaptation

Adaptation by gene duplication has been observed in bacteria, yeast, cancer cells, and plant cell cultures (Hyppa and Smith 2010; Slack et al. 2006; Suh et al. 1993; Watanabe et al. 2011). There are many reasons why gene duplications and CNV are a frequent mechanism underpinning adaptation. All genes contained within the region have increased expression, which may be adaptive, but not all genes

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necessarily have immediate changes in function. All genes within the region maintain their own promoters and all cis-regulatory elements used to modulate their expression. Due to redundancy in function, one or more of the gene copies is free from selection pressure to diverge through random mutations, assuming at least one copy maintains the original function. This divergence usually ends in pseudogenes but may also result in neo- or sub-functionalization, thereby generating novel genetic diversity which may be adaptive (Flagel and Wendel 2009; Lynch and Conery 2000).

Silent point mutations in the genome are a fairly consistent molecular clock and non-silent point mutations that change protein function are often subject to purifying selection (Drake et al. 1998). The rate of CNV generation, on the other hand, is variable and is subject to environmental factors. Under more intense selection pressures the number of CNV events in offspring increases, while under optimal conditions fewer genomic rearrangements are observed (DeBolt 2010). Species which have evolved higher rates of CNV, or more sensitivity to stress, may have increased genetic diversity, and therefore an increased chance of survival under strong selective pressures such as herbicide application (Kondrashov 2012; Żmieńko et al. 2014). This type of heritable, possibly adaptive, genetic variation due to CNV is especially important in plants that have short generational timescales and live in constantly changing environments with strong selective pressures (such as weeds) (DeBolt 2010; Hastings et al. 2009). The prevalence of CNV underlying glyphosate resistance provides further support for the importance of this mode of adaptation.

Gene amplification has been shown in arthropods to cause insecticide and miticide resistance for almost thirty years (Bass and Field 2011; Devonshire and Field 1991). A general expansion and functional diversification within gene families via gene duplication is evident in the genomes of pest species such as *Anopheles gambiae* when compared to *Drosophila melanogaster* (Ranson et al. 2002). In arthropods, gene amplification typically results in the overexpression of certain metabolic genes, including esterase (Hemingway 2000; Hemingway et al. 1998; Li et al. 2007; Ono et al. 1999; Raymond

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et al. 1989; Small and Hemingway 2000), glutathione-*S*-transferase (Vontas et al. 2001; Zhou and Syvanen 1997), and cytochrome P450 monooxygenase (Emerson et al. 2008; Schmidt et al. 2010). However, the target gene of insecticides and miticides can also be amplified and over-expressed to cause resistance, similar to the case of *EPSPS* gene duplication (Anthony et al. 1998; Kwon et al. 2010; Labbé et al. 2007b).

In the case of organophosphate resistance in *Culex pipiens*, the target gene acetylcholinesterase is duplicated and one of the copies carries a point mutation that generally confers a severe fitness cost. However, one copy maintains the wild-type sequence and continues to function normally, while the mutant copy confers a resistance benefit in the presence of the insecticide. In effect this series of genetic mutations (copy number variation followed by a single base pair mutation) has effectively resulted in a permanent heterozygous genotype with different alleles in duplicated genes (Bourguet et al. 1997; Labbé et al. 2007a; Labbé et al. 2007b). While this is an interesting example of how copy number variation can confer resistance, a more recent example in *Tetranychus urticae* links the number of copies of the target genes in a directly proportional relationship to the amount of target protein produced. Because the pool of target protein is larger, the amount of active ingredient needed to inhibit the protein pool also must increase, thereby conferring resistance to higher doses of organophosphate miticides (Kwon et al. 2010).

In animals (especially humans) copy number variation is often associated with genetic disorders, especially cancer; however, in plants there exist several examples of how copy number variations can generate genetic diversity useful for adaptation (Mishra and Whetstine 2016). In plants, resistance to the soybean root knot nematode in some soybean cultivars is due to duplication of three genes, resulting in over-expression of the three genes that is directly correlated with nematode resistance (Cook et al. 2012). Another example of the adaptive potential of CNVs is in clonally propagated potato which shows prolific and genome wide copy number variation. Clonally propagated varieties have upward of 30% of the genes in the genome duplicated or deleted. Additionally, there is a specific increase in the number of genes

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annotated as having roles in environmental stress tolerance. It is thought that clonally propagated plants tolerate a larger mutational load as they do not need to undergo meiosis and produce seed, both of which can be negatively affected by genomic rearrangements (Hardigan et al. 2016). Copy number variations may provide plants with novel genetic diversity, and their production may be stimulated by stress.

Recently resistance to Acetyl-CoA Carboxylase (ACCase)-inhibiting herbicides in hairy crabgrass (*Digitaria sanguinalis*) was reported to be due to 5 to 7-fold increase in *ACCase* gene copy number resulting in 3 to 9-fold increase in *ACCase* transcript abundance (Laforest et al. 2017). This provides the first example of CNV for resistance to a herbicide other than glyphosate, and further highlights the potential advantages of adaptive CNVs for rapidly generating increased gene expression phenotypes to confer herbicide resistance. Other than this recent example, to date gene duplication as a herbicide resistance mechanism has only been identified for *EPSPS* and glyphosate resistance, a target-site mechanism. This raises the question as to why there is a prevalence of the CNV-based mechanism for glyphosate. The *EPSPS* CNV may be an extremely rare event that is only revealed by intense selection over large geographical areas. Perhaps the genomic context of *EPSPS* happens to be more prone to duplication than other herbicide target-site genes, enabling tandem duplication and/or transduplication. The relatively low resistance level conferred by single nucleotide mutations in *EPSPS* (reviewed by Sammons and Gaines 2014) and the apparent high fitness cost of the highly-resistant double mutation T102I and P106S in *EPSPS* (TIPS) (Vila-Aiub et al. 2017; Yu et al. 2015) may indicate that *EPSPS* over-expression by gene duplication is a more efficient mechanism, in contrast to several other herbicide target genes for which target-site mutations are highly efficient and commonly selected (Powles and Yu 2010). However, the P106S mutation was recently shown to have a fitness advantage over *EPSPS* gene duplication in waterhemp, as the P106S mutation increased in frequency over six generations without glyphosate selection while the *EPSPS* CNV decreased in frequency (Wu et al. 2017). Additionally, previous research may have simply failed to consider gene duplication as a possible resistance mechanism, resulting in CNVs being overlooked in some cases of herbicide resistance evolution.

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Resistance to some herbicides is known to be caused by increased expression of non-target-site genes that metabolize the herbicide, including glutathione S-transferase (Cummins et al. 2013) and cytochrome P450 monooxygenase (Duhoux et al. 2015; Gaines et al. 2014; Gardin et al. 2015; Iwakami et al. 2014). In general the examples of increased non-target-site gene expression have not yet been evaluated for CNV.

Summary

To date, four dicotyledon species and four monocotyledon (grass) species have evolved *EPSPS* gene amplification resulting in glyphosate resistance. One of those species, spiny amaranth, obtained high copy numbers by interspecific gene flow while the other seven species seem to have evolved *EPSPS* gene amplification independently in a case of convergent evolution. In one species, Palmer amaranth, the mechanism of gene duplication is partially understood, involving transduplication of >300 kb of sequence containing EPSPS to multiple novel insertion sites, possibly through a helitron-like mechanism. Gene amplification in kochia is also well studied, occurring by a different mechanism with extra gene copies arranged as tandem duplications likely caused by unequal crossing over. In the remaining species, further investigation is required to elucidate the mechanisms that generated *EPSPS* gene amplification.

The convergent evolution of the same resistance mechanism, increased *EPSPS* gene copy number, via two different genomic mechanisms is quite striking and raises several questions. 1) Is *EPSPS* gene amplification present at initially low frequencies (i.e., rare standing genetic variation for *EPSPS* CNV) and how often does *EPSPS* gene amplification occur due to normal DNA repair processes or mobile genetic element activity (i.e., *de novo* genetic variation)? 2) Are potential fitness costs associated with *EPSPS* gene amplification, whether physiological (consequences of over-expressing *EPSPS* and/or other duplicated genes), genomic (disruption of other genes when the *EPSPS* replicon inserts at a novel locus), or energetic (increased ATP and amino acid usage to produce an over-abundance of EPSPS enzyme) likely to be balanced by ongoing selection for maximum resistance benefit with minimal fitness cost? 3) Given the previously observed instability of increased *EPSPS* gene copy number in plant cell

culture and the instability of other gene duplications for xenobiotic resistance (e.g., in cancer cells), would *EPSPS* gene amplification be retained if glyphosate selection pressure were removed, and does the stability depend on the genomic mechanism (tandem duplication or dispersed transduplications)? 4) What genetic and genomic mechanisms underlie the production of high *EPSPS* copy numbers in these eight species ? 5) Why has *EPSPS* gene duplication been observed to date only in the Amaranthaceae and Poaceae plant families? 6) Are CNVs more likely to arise independently in different populations of the same species, than to migrate via gene flow? The convergent evolution of CNVs for glyphosate resistance in weeds, through at least two mechanisms, may be indicative of a more general importance for this mechanism of adaptation in plants. CNVs warrant further investigation across plant functional genomics for adaptation to biotic and abiotic stresses, particularly for adaptive evolution on rapid time scales.

Funding

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