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Deciphering the Mechanism of Glyphosate Resistance in *Amaranthus palmeri* by Cytogenomics

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Keywords

Amaranthus palmeri · Cytogenomics · eccDNA · Glyphosate resistance · Tethering

Abstract

In agriculture, various chemicals are used to control the weeds. Out of which, glyphosate is an important herbicide invariably used in the cultivation of glyphosate-resistant crops to control weeds. Overuse of glyphosate results in the evolution of glyphosate-resistant weeds. Evolution of glyphosate resistance (GR) in *Amaranthus palmeri* (AP) is a serious concern in the USA. Investigation of the mechanism of GR in AP identified different resistance mechanisms of which *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene amplification is predominant. Molecular analysis of GR AP identified the presence of a 5- to >160-fold increase in copies of the *EPSPS* gene than in a glyphosate-susceptible (GS) population. This increased copy number of the *EPSPS* gene increased the genome size ranging from 3.5 to 11.8%, depending on the copy number compared to the genome size of GS AP. FISH analysis using a 399-kb *EPSPS* cassette derived from bacterial artificial chromosomes (BACs) as probes identified that amplified *EPSPS* copies in GR AP exist in extrachromosomal circular DNA (eccDNA) in addition to the native copy in the chromosome. The *EPSPS* gene-containing eccDNA having a size of ~400 kb is termed *EPSPS*-eccDNA and showed somatic mosacism in size and copy number. *EPSPS*-eccDNA has a genetic mechanism to tether randomly to mitotic or meiotic chromosomes during cell division or gamete formation and is inherited to daughter cells or progeny generating copy number variation. These eccDNAs are stable genetic elements that can replicate and exist independently. The genomic characterization of the *EPSPS* locus, along with the flanking regions, identified the presence of a complex array of repeats and mobile genetic elements. The cytogenomics approach in understanding the biology of *EPSPS*-eccDNA sheds light on various characteristics of *EPSPS*-eccDNA that favor GR in AP.

Introduction

Amaranthus palmeri (AP) is an annual herbaceous plant, which is a troublesome and economically damaging agronomic weed in the agricultural production system of the USA. Chemical control of AP offers a cost-ef-

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fective control, however, overuse of and sole reliance on glyphosate for AP control has resulted in the evolution of glyphosate resistance (GR) in AP [Duke and Powles, 2008]. Modelling predicts that 5 applications of glyphosate each year with no other herbicides would result in resistance evolving in 74% of the simulated AP populations [Neve et al., 2011]. The primary mechanism of GR in AP is the amplification of the *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene [Gaines et al., 2010]. A similar GR mechanism was reported in Italian ryegrass [Salas et al., 2012], Kochia [Jugulam et al., 2014; Wiersma et al., 2015], waterhemp [Lorentz et al., 2014; Chatham et al., 2015], goose grass [Chen et al., 2015], ripgut brome [Malone et al., 2016], and windmill grass [Ngo et al., 2018]. FISH, a molecular cytogenetic method, was used to study the mechanism of *EPSPS* gene amplification in AP [Gaines et al., 2010], kochia [Jugulam et al., 2014], and waterhemp [Dillon et al., 2017]. With the advances in sequencing technologies and the availability of robust bioinformatics platforms, cytogenetic approaches combined with genomic approaches have given rise to cytogenomics, uniting ideas from classical, new cytogenetic, molecular-genetic/genomic and bioinformatic approaches under one roof [Liehr 2021]. Cytogenomics is a very powerful approach to address GR in AP [Gaines et al., 2010; Molin et al., 2017; Koo et al., 2018]. The first report that amplified copies of the *EPSPS* gene in GR in AP are present in the form of extrachromosomal circular DNA molecules (eccDNAs) conferring GR was given by Koo et al. [2018].

The concept that DNA might be organized as series of rings in the chromosomes of higher organisms was put forth by Franklin Stahl [Cairns, 1963] and experimentally proved by Hotta and Bassel [1965]. They referred to the circles of DNA of various sizes in mammalian cells as Double Minutes (DMs). In eukaryotes, eccDNA size ranges from <100 bases up to several hundred thousand base pairs. Based on the different sizes and sequences of eccDNA, Liao et al. [2020] categorized them into small polydispersed DNA (spcDNA; 100 bp–10 kb), telomeric circles (t-circles; multiples of 738 bp), microDNA (100–400 bp), and extrachromosomal DNA (ecDNA; the largest one with millions of bp). Based on the cell type, tissue type, and genetic background, these DNA molecules show heterogeneity in size and copy number. eccDNAs can replicate and propagate in cells as stable genetic elements [Shoura et al., 2017; Turner et al., 2017]. eccDNA may originate from nuclear DNA by homologous recombination between adjacent repeats, such as amplified genes [Mukherjee and Storici, 2012] or tandem repeats (satellite, telomeric, centromeric, and ribosomal repeats) [Cohen et al., 2008; Diaz-Lara et al., 2016] or they can result from linear extrachromosomal forms of active transposable elements [Gaubatz 1990]. In plants, eccDNA derived from satellite repeats are common [Navratilova et al., 2008]. In eukaryotes, eccDNAs contain not only complete or partial genes but also intergenic sequences [Møller et al., 2018; Yerlici et al., 2019], playing a crucial role in genome evolution in response to selection pressure [Gresham et al., 2010]. eccDNAs can serve as templates for the reinsertion of amplified genes into chromosomes in yeast [Demeke et al., 2015]. In this review, a case study of eccDNA-mediated GR in AP is discussed.

Genomics of Glyphosate Resistance in *A. palmeri*

Ten years after the introduction of GR crops in the USA, the first case of GR in an AP population was reported in Georgia [Culpepper et al., 2006; Molin et al., 2017]. Various molecular mechanisms conferring GR in AP were reported [Gaines et al., 2011]. The GR AP population from Macon County, Georgia, was used to investigate the mechanism of resistance. The GR AP population contained 5- to >160-fold copies of the *EPSPS* gene than the glyphosate-susceptible (GS) population [Gaines et al., 2010]. Such a huge copy number increase contributed to the increase in genome size of GR AP [Molin et al., 2017]. A GS AP population from Iowa had a genome size of 0.82 pg/2C, whereas GR AP from Mississippi had various genome sizes depending on the number of copies of the *EPSPS* gene. Plants having 77, 79, 92, and 106 copies of the *EPSPS* gene had genome sizes of 0.88 pg/2C (6.8% genome increase), 0.85 pg/2C (3.5% genome increase), 0.87 pg/2C (5.75% genome increase), and 0.93 pg/2C (11.8% genome increase), respectively [Molin et al., 2017]. FISH analysis using the *EPSPS* gene as a probe revealed that amplified *EPSPS* gene copies were dispersed in every chromosome throughout the genome, suggesting that *EPSPS* gene amplification in GR AP was not caused by unequal crossing over or rolling circle replication-based mechanisms. Gaines et al. [2010] speculated that *EPSPS* amplification in GR AP could have originated via transposon-mediated elements that were activated during stress. He put forward the hypothesis that "the original *EPSPS* locus was associated with a mobile genetic element that activated and amplified the *EPSPS* gene". Following this hypothesis, Gaines et al. [2013] constructed a fosmid library and generated a ~30-kb se-

quence harboring 10 kb of the *EPSPS* gene flanked by a 1.5-kb upstream and 20-kb downstream region. Analysis of the genomic regions around the *EPSPS* gene revealed that it is bordered by miniature, inverted-repeat transposable elements (MITEs) and a putative Activator (Ac) transposase [Gaines et al., 2013].

To explore more genomic regions around the *EPSPS* gene, Molin et al. [2017] constructed a BAC tiling path covering the *EPSPS* locus and flanking regions in GR AP, which was sequenced using the PacBio platform. The de novo assembly of the sequence produced a final sequence length of 297,445 bp. This *EPSPS* cassette contained a single copy of the *EPSPS* gene flanked by nearly equal-sized upstream and downstream regions. Around 300 repeat sequences were identified in the *EPSPS* cassette, predominately simple repeats, followed by Class I and II transposons. Helitrons, a novel class of repeats associated with genome reshuffling and gene amplification, were detected downstream of the *EPSPS* gene [Molin et al., 2017]. Based on the genomic characterization of the upstream and downstream regions of *EPSPS* in AP, the structural genomic region of the *EPSPS* locus is amplified and distributed among all chromosomes mediated by transposons in GR AP, which was further supported by FISH analysis [Gaines et al., 2010]. However, the notion that the *EPSPS* cassette is integrated randomly into chromosomes through the action of transposons became untenable when fiber-FISH conclusively demonstrated that it is a distinct entity organized as an extrachromosomal circular DNA that mediated the amplification of the *EPSPS* gene in GR AP [Koo et al., 2018].

EPSPS-eccDNA in Glyphosate Resistant *A. palmeri*

EccDNA containing the *EPSPS* gene in GR AP is termed *EPSPS*-eccDNA and is ca. 400 kb in size [Koo et al., 2018; Molin et al., 2020a]. Predominately, plant eccDNAs contain tandem repeats, which supports the concept of the origin of *EPSPS*-eccDNA by intrachromosomal homologous recombination. In natural selection, the mechanism for controlling the length of a nucleotide string is favored by the formation of tandem-repetitive structures rather than the sequence itself [Stephan and Cho, 1994]. Formation of eccDNA from tandem repeats was reported in *Drosophila* [Pont et al., 1987; Cohen et al., 2003], *Xenopus* [Cohen and Mechali, 2001], *Arabidopsis* [Navratilova et al., 2008; Wang et al., 2021], *Oryza*, *Pisum*, *Secale*, *Triticum,* and *Vicia* [Navratilova et al., 2008]. Hence, the predominance of complex arrays of repeats and transposon sequences [Molin et al., 2017] in *EPSPS*-eccDNA likely plays a role in controlling the size and dynamics of *EPSPS*-eccDNA in GR AP. Sequences containing 143 $(AAAT)_2$ or 186 $(AATT)_2$ motifs flank the *EPSPS* gene in *EPSPS*-eccDNA. In addition, *EPSPS*-eccDNA contains clustered long and short interspersed palindromic repeat sequences (CLiSPrs) flanking the *EPSPS* gene. These repeats may play a crucial role in the stability and dynamics of *EPSPS*-eccDNA [Molin et al., 2020a]. The *EPSPS*-eccDNA also have long terminal repeat (LTR) retroposons and non-LTR retroposons, which amplify by a copy and paste mechanism. Generally, LTRs carry a response element that directs the onset and termination of retrotransposon replication. Plant LTR promoters are activated by a variety of biotic and abiotic stresses [Ansari et al., 2007; Salazar et al., 2007]. In the life cycle of an LTR retroposon, the formation of eccDNA is a byproduct, which is an indicator for their continuing activity [Lanciano et al., 2017]. EccDNA is produced through heat-responsive, copia-like ONSEN retrotransposons in *Arabidopsis* and drug-induced activation of the Houba retrotransposons in *Oryza sativa* [Thieme et al., 2017]. The presence of repeats and transposons upstream and downstream of the *EPSPS* locus may facilitate the formation of *EPSPS*-eccDNA as a response to natural selection, conferring GR in AP.

Stability and Inheritance of *EPSPS*-eccDNAs

The first cytological evidence of *EPSPS*-eccDNA tethering to chromosomes mediating their inheritance to the daughter cells during cell division in plants was reported by Koo et al. [2018]. Typically, *EPSPS*-eccDNAs are randomly transmitted to daughter cells due to the lack of centromeres. The *EPSPS*-eccDNAs are transmitted to the next generation by tethering to meiotic chromosomes leading to a rapid spread and development of GR [Koo et al., 2018; Fig. 1]. The *EPSPS*-eccDNAs are not integrated back into the chromosome and exist as autonomous replicating structures that display unequal mitotic segregation and, thereby, produce soma cell heterogeneity for evolution of GR [Koo et al., 2018]. The sequencing and annotation of *EPSPS*-eccDNA revealed genetic elements that are responsible for *EPSPS*-eccDNA stability, replication, and tethering. Molin et al. [2020a] predicted that *AP_R.00g000496* encodes a protein containing a helicase domain, which is involved in DNA replication and tethering of *EPSPS*-eccDNA to the nuclear chromatin. Molin et al. [2020b] described *EPSPS*-eccD-

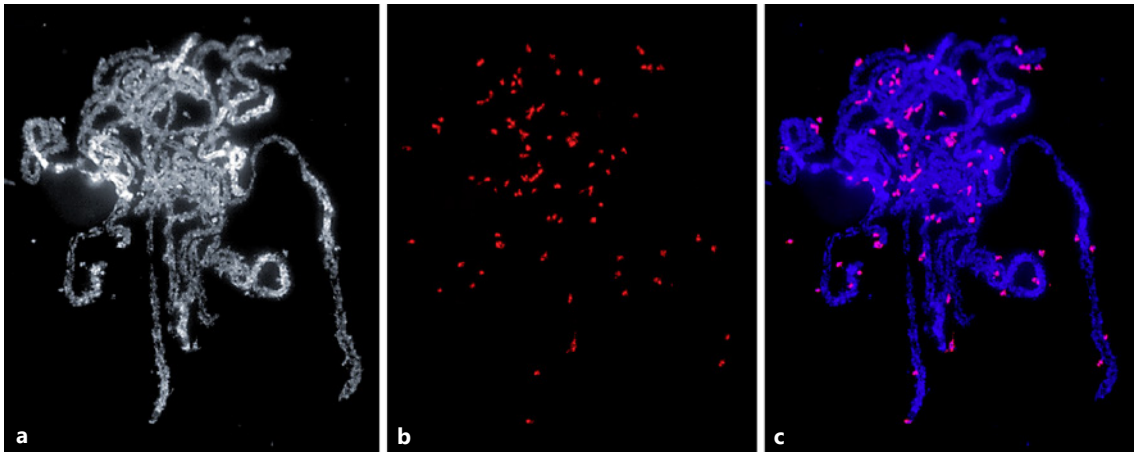

Fig. 1. FISH mapping of *EPSPS*-eccDNA (red signals) on meiotic pachytene chromosomes of glyphosate-resistent *A. palmeri* with 80 *EPSPS* copies. **a** DAPI-stained pachytene chromosome showing *EPSPS*-eccDNAs lying outside the pachytene chromosomes. **b** FISH signals with the *EPSPS*-eccDNA probe. **c** Merged image.

NA as an autonomously replicating genetic element possessing autonomous replication sequences (ARS) and having homology with the ARS sequence found in yeast and other eukaryotes. The ARS sequence in AP contains 2 putative DNA unwinding elements rich in A + T content and displaying helical instability that can result in a unique DNA structure that can facilitate accessibility to the DNA replisome. The origin of replication (ori) on the eccDNA replicon occurs in a putative NAC-containing gene. The ARS sequence of the eccDNA replicon from AP enabled the autonomous replication in ARS-less yeast plasmid.

Structural Conformation and Copy Number Variation

Fiber FISH analysis using 6 BACs representing the 399-kb *EPSPS* cassette [Koo et al., 2018] revealed the existence of structural polymorphisms in *EPSPS*-eccDNA. Predominately, *EPSPS*-eccDNA exist in circular (50.2%), followed by linear (21.8%), dimeric circular (11.8%), dimeric linear (8%), and atypical (8.2%) forms. The circularity of the *EPSPS*-eccDNA accounts for the stability and integrity of *EPSPS*-eccDNA, where the closed circular structure of eccDNA may impart resistance to exonuclease digestion. The purpose of structural polymorphism in *EPSPS*-eccDNA is not well understood. However, characterizing *EPSPS*-eccDNA structural polymorphisms may give clues regarding the pathway that gives rise to *EPSPS*-eccDNA. The copy number of *EPSPS*-eccDNA varies between the somatic cells, generating somatic mosaicism patterns. Using 2-color FISH with 5S rDNA and *EPSPS*-eccDNA as probes, 4 different patterns of *EPSPS*-eccDNA signals on 5S rDNA-labeled homologous chromosome pairs in different cells derived from a single root tip meristem were observed: (1) both chromosomes lack *EPSPS*-eccDNA signals (16.7%), (2) 1 of the 2 chromosomes lacks an *EPSPS*-eccDNA signal (25%), (3) both chromosomes have a similar signal intensity (33.3%), and (4) the 2 chromosomes vary in signal intensity (25%) [Koo et al., 2018]. We hypothesize that *EPSPS*-eccDNA is not associated with chromosomes during the interphase stage. *EPSPS*-eccDNAs in metaphase tend to randomly anchor to chromosomes and are associated with the chromosomes until the end of telophase, enabling their transmission to the daughter cells leading to somatic mosaicism (Fig. 2). *EPSPS*-eccDNAs also are inherited by tethering to meiotic chromosomes and confer copy number variation in somatic cells of the progeny. From metaphase cell spreads from a single root preparation of one plant, we observed that (1) *EPSPS*-eccDNAs were associated with most of the chromosomes similar to the GR AP, (2) *EPSPS*-eccDNAs were associated with half of the chromosomes, (3) *EPSPS*-eccDNAs were associated with only a few chromosomes, and (4) all chromosomes were free of *EPSPS*-eccDNAs. Similarly, a huge variation in *EPSPS*-eccDNA distribution in the cells was observed when analyzing the F_1 progeny derived from the cross between GS AP and GR AP [see Fig. 5 shown in Koo et al., 2018]. This copy number variation is due

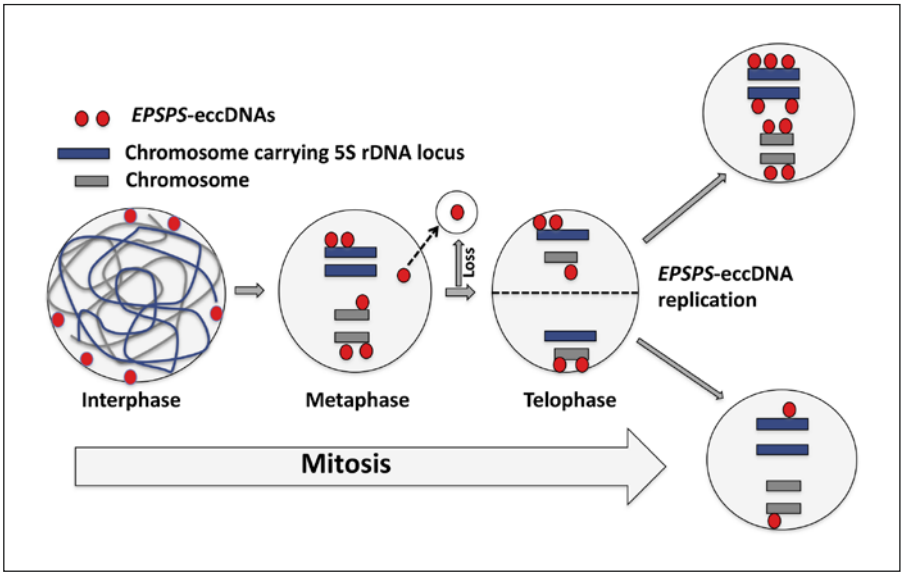

Fig. 2. Pictorial representation of *EPSPS*-eccDNA behaviours during mitotic cell division. *EPSPS*-eccDNAs in interphase are not associated with chromosome. *EPSPS*-eccDNA are associated with chromosome from metaphase to telophase, and *EPSPS*-eccDNA not associated with a chromosome will be lost during anaphase.

to the independent existence of *EPSPS*-eccDNA and its unequal segregation during mitotic divisions.

EPSPS-eccDNA Chromatin Landscape

Genes that occur in eccDNA amplify much faster than those in chromosomes. Turner et al. [2017] speculated that the genes carried by eccDNA can be easily accessed by the transcription machinery compared with chromosomal genes. Wu et al. [2019] further demonstrated that eccDNA was packaged into chromatin with an intact domain structure but lacked the higher-order compaction that is typical of chromosomes and displayed significantly enhanced chromatin accessibility enabling high transcription of genes. Similarly, preliminary investigation of eccDNA from AP observed that *EPSPS*-eccDNAs, highly condensed in mitotic and meiotic metaphase I chromosomes, are barely visible by DAPI staining [see Fig. 1 shown in Koo et al., 2018]. In meiotic pachytene stage, where chromosomes are decondensed and highly elongated, *EPSPS*-eccDNA appear as large dots [see Fig. 3 shown in Koo et al., 2018]. This correlation of chromosome condensation progression from meiotic pachytene through metaphase stages suggests that the *EPSPS*-eccDNA of AP may consist of basic nucleosome units organized into chromatin, rather than as naked DNA, and needs further investigation. Characterizing the histones present in *EPSPS*-eccDNAs will shed light on the structure and organization of *EPSPS*-eccDNA.

Conclusion

The introduction of GR crops favored the agriculture production system for a short period. Extensive use of glyphosate for the control of weeds gave rise to GR weeds. Different mechanisms operate that confer GR in plants. A case study with GR AP demonstrated how cytogenomics can unravel the mystery of evolution of GR. Genomic characterization of a 399-kb *EPSPS* cassette derived from BACs identified the complex array of repeats and the transposons flanking the *EPSPS* locus. Cytological investigation with BACs representing the *EPSPS* cassette identified that the *EPSPS* in GR AP exist as eccDNA, in addition to the *EPSPS* gene at the native locus of the chromosome. eccDNA containing the *EPSPS* gene in AP is termed *EPSPS*-eccDNA and exists as an autonomously replicating genetic element able to transmit during cell division by tethering to the mitotic chromosome. *EPSPS*-eccDNA is inherited to the progeny by tethering to meiotic chromosomes during gamete formation. This *EPSPS*-eccDNA is highly heterogeneous, exhibiting both copy number variation and somatic mosaicism. This copy number variation is attributed to unequal *EPSPS*-eccDNA segregation during mitotic divisions. The role of *EPSPS*-eccDNA in conferring genome plasticity to glyphosate challenge is discussed.

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Conflict of Interest Statement

The authors report no competing interests.

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Author Contributions

D.-H.K., R.S., B.F., and B.S.G. wrote the manuscript and analyzed the data and helped to draft the final manuscript.

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