https://doi.org/10.1093/plphys/kiad281 PLANT PHYSIOLOGY 2023: 1–5

Plant Physiology®

Research Report

Extrachromosomal circular DNA–mediated spread of herbicide resistance in interspecific hybrids of pigweed

Dal-Hoe Koo [1,*] Rajendran Sathishraj [1] Sridevi Nakka [2] Yoonha Ju [1] Vijay K. Nandula [3,4] Mithila Jugulam [5] Bernd Friebe [1] and Bikram S. Gill [1]

1 Wheat Genetics Resource Center and Department of Plant Pathology, Kansas State University, Manhattan, KS 66506, USA
2 Heartland Plant Innovations Inc., Manhattan, KS 66506, USA
3 National Institute of Food and Agriculture, USDA, Kansas City, MO 64105, USA
4 Crop Production Systems Research Unit, USDA-ARS, Stoneville, MS 38776, USA
5 Department of Agronomy, Kansas State University, Manhattan, KS 66506, USA

*Author for correspondence: dkoo@ksu.edu
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/General-Instructions) is: Dal-Hoe Koo (dkoo@ksu.edu).

Abstract

Extrachromosomal circular DNAs (eccDNAs) are found in many eukaryotic organisms. EccDNA-powered copy number variation plays diverse roles, from oncogenesis in humans to herbicide resistance in crop weeds. Here, we report interspecific eccDNA flow and its dynamic behavior in soma cells of natural populations and F_1 hybrids of *Amaranthus* sp. The glyphosate-resistance (GR) trait is controlled by eccDNA-based amplification harboring the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene (eccDNA replicon), the molecular target of glyphosate. We documented pollen-mediated transfer of eccDNA in experimental hybrids between glyphosate-susceptible *Amaranthus tuberculatus* and GR *Amaranthus palmeri*. Experimental hybridization and fluorescence in situ hybridization (FISH) analysis revealed that the eccDNA replicon in *Amaranthus spinosus* derived from GR *A. palmeri* by natural hybridization. FISH analysis also revealed random chromosome anchoring and massive eccDNA replicon copy number variation in soma cells of weedy hybrids. The results suggest that eccDNAs are inheritable across compatible species, contributing to genome plasticity and rapid adaptive evolution.

Introduction

Extrachromosomal circular DNA (eccDNA) is generated from chromosomal DNAs in sizes ranging from a few hundred base pairs up to megabases and contributes to genome plasticity in eukaryotes (Stark and Wahl 1984; Cohen et al. 2008; Pennisi 2017; Koo et al. 2018). High copy number and expression of eccDNA drive the oncogenesis and promote the survival and proliferation of cancerous cells in humans (Turner et al. 2017). In plants, Koo et al. (2018) reported that glyphosate resistance (GR) in the troublesome crop weed *Amaranthus palmeri* was driven by eccDNA containing the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene, the molecular target of glyphosate (Steinrücken and Amrhein 1980). This specific eccDNA is hereafter referred to as eccDNA replicon. The eccDNA replicon was sequenced by Molin et al. (2020). The sequence characterization of eccDNA replicon from geographically distant populations revealed that the eccDNA replicon may have a common origin and be transmitted from one population to another (Spier-Camposano et al. 2022).

GR may involve target-site alterations, either by mutation or by amplification of the *EPSPS* gene (Gaines et al. 2010; Dillon et al. 2017). The amplification of the *EPSPS* gene conferring GR in *A. palmeri* was documented in Georgia in 2006

Received March 01, 2023. Accepted April 18, 2023. Advance access publication May 15, 2023

Open Access

and this resistance has since spread to most states in the United States. All analyzed GR populations carry the identical eccDNA replicon (Koo et al. 2018; Molin et al. 2018). Nandula et al. (2014) reported the apparent transfer of GR from *A. palmeri* to the related species *Amaranthus spinosus* L., another troublesome weed. The analysis of GR *A. spinosus* genotypes showed amplification of the *EPSPS* gene (up to 40 copies), and their gene sequence was identical to the *EPSPS* gene of GR *A. palmeri* (Nandula et al. 2014). This finding led to the hypothesis that GR found in *A. spinosus* arose from pollen-mediated eccDNA replicon transfer from GR *A. palmeri*. Our results confirm this hypothesis. Furthermore, we demonstrate that the eccDNA replicon drives the spread and rapid evolution of herbicide resistance in an interspecific hybrid of GS *Amaranthus tuberculatus* (female, $2n = 32$) and GR *A. palmeri* (male, $2n = 34$).

Results

Pollen-mediated eccDNA replicon flow between species

Fiber-fluorescence in situ hybridization (FISH) analysis using six bacterial artificial chromosomes (BACs) (used in Koo et al., 2018) associated with eccDNA replicon of GR *A. palmeri* detected 2 types of eccDNA replicons in GR *A. spinosus* (Fig. 1), circular and linear with structural polymorphisms similar to those found in GR *A. palmeri* (Koo et al. 2018). The results indicated transfer of eccDNA replicon from GR *A. palmeri* to GR *A. spinosus via* pollen by interspecific hybridization in the natural environment. To test further the pollen-mediated eccDNA replicon transfer by interspecific inheritance, 6 interspecific F_1 hybrids were generated from a cross between *A. tuberculatus* (female, $2n = 32$) and GR *A. palmeri* (male, $2n = 34$) carrying eccDNA replicon. Molecular analysis of 6 F_1 hybrids using species-specific markers revealed that 2 F_1 hybrids (W-P#3 and W-P#6) were positive for both the markers tested, while the other 4 F_1 hybrids were negative for the *A. palmeri*–specific marker (Fig. 2A). All the 6 F_1 hybrids were positive for the *A. tuberculatus*–specific marker (Fig. 2B).

Random chromosome tethering and somatic mosaicism for copy number variation of eccDNA replicon in F_1 hybrids

FISH analysis detected 5 pairs of chromosomes carrying the 5S rDNA signals in *A. tuberculatus* (Fig. 2C) and 1 pair in *A. palmeri* (Fig. 2D). As expected, the 5S rDNA-FISH detected 6 major hybridization signals in the metaphase chromosomes of the F_1 hybrid, W-P#3; 5 from *A. tuberculatus* and 1 from *A. palmeri* (Fig. 2, E to G). The 5S rDNA-FISH marker chromosomes provided an opportunity to study the dynamics of eccDNA replicon anchoring to chromosomes of interspecific origin. The eccDNA replicon FISH signals were randomly associated with 5S rDNA-marker chromosome of *A. tuberculatus* in the F_1 hybrid plant, W-P#3. One cell had eccDNA replicon tethered with 4 of the 5 chromosomes of *A. tuberculatus* and with 1 chromosome of *A. palmeri* (Fig. 2E). The second cell had eccDNA replicon tethered with only 1 of the 5 chromosomes of *A. tuberculatus* and with 1 chromosome of *A. palmeri* (Fig. 2F). The third cell had no tethering of eccDNA replicon with any of the 5S rDNA-marker chromosomes (Fig. 2G). The analysis provided conclusive

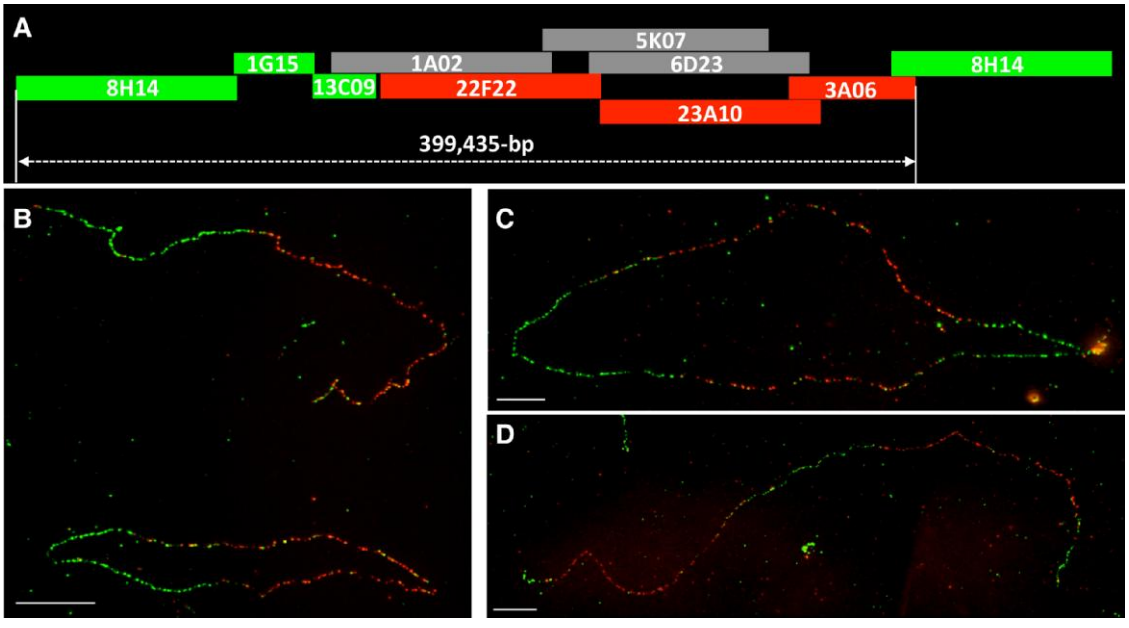

Figure 1. Fiber-FISH images of eccDNA replicons in GR *A. spinosus*. **A)** Schematic representation of BAC-contig assembly of eccDNA replicon used in fiber-FISH analysis (Koo et al. 2018); **B)** a circular form (bottom) and linear form of eccDNA replicons (top); **C)** dimerized circular form of eccDNA replicon with head-to-tail tandem orientation; **D)** linear form of eccDNA replicon with head-to-tail dimer. Green: BAC 8H14, BAC 1G15, BAC 13C09. Red: BAC 22F22, BAC 23A10, BAC 3A06. Bars = 10 μm.

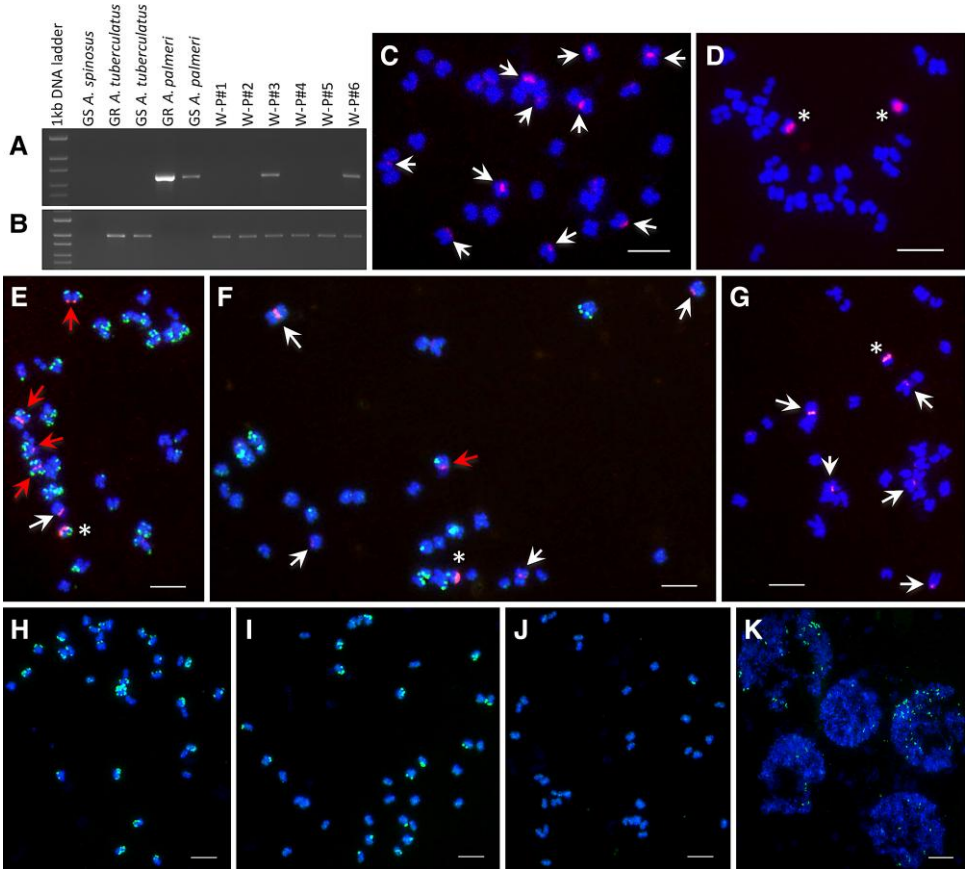

Figure 2. Conformation of F_1 hybrids by PCR-based amplification and characterization of F_1s along with their parents by FISH. Amplification of *A. palmeri*–specific 697 bp genomic region using AW155/AW90 markers **A)**. Amplification of *A. tuberculatus*–specific 992 bp genomic region using AW468/AW469 markers **B)**. Two F_1 hybrids (W-P#3 and W-P#6) were positive for both the markers tested. FISH mapping of 5S rDNAs on mitotic metaphase chromosomes of *A. tuberculatus* ($2n = 32$; white arrows in **C**) and *A. palmeri* ($2n = 34$; white asterisks in **D**). Distribution of eccDNA replicons (green signals) on the 5S rDNA-marker chromosomes of *A. tuberculatus* in F_1 hybrid from single root tip **E to G**). Out of 5 5S rDNA-marker chromosomes of *A. tuberculatus* (arrows) 4 (red arrows), **E)** 1 **F)**, and none **G)** of them had eccDNA replicons. The 5S rDNA-marker chromosome from *A. palmeri* is marked by an asterisk. Distribution of eccDNA replicons (green signals) on the chromosomes of GR *A. spinosus* from single root tip **H to K**). eccDNA replicons (green signals) associated with most **H)**, half **I)**, and few **J)** of the mitotic metaphase chromosomes of GR *A. spinosus* and mosaic pattern of eccDNA replicon copy numbers in interphase cells of the same tissue **K)**. Bars = 5 μm.

evidence that the eccDNA replicon of *A. palmeri* is capable of random tethering to chromosomes of a related species.

Apart from random tethering, somatic mosaicism for copy number variation of eccDNA replicon was observed in GR *A. spinosus* and F_1 hybrids. In a sample of 3 cells of GR *A. spinosus* (Fig. 2, H to J), 1 cell had eccDNA replicon signals on most of the chromosomes (Fig. 2H; Pattern I), the second cell had eccDNA replicon signals on half of the chromosomes (Fig. 2I; Pattern II), and the third cell had few eccDNA replicon signals (Fig. 2J; Pattern III). The frequency of Patterns I, II, and III observed in a sample of 50 cells of GR *A. spinosus* having *EPSPS* gene copy number of 54.3 ± 2.3 (Table 1) was 30%, 54%, and 2%, respectively. In F_1 hybrid plant W-P#3 (Fig. 2, F and G; $n = 30$) where the *EPSPS* gene copy number was 3.0 ± 0.8 (Table 2), the observed frequency of eccDNA replicon signals corresponding to Patterns I, II, and III was 20%, 66.7%, and 13%, respectively. Similar cytological behavior of eccDNA replicon was also observed in the W-P#6 genotype (Table 2).

Discussion

Nandula et al. (2014) reported that GR in *A. spinosus* was due to amplification of the *EPSPS* gene. The *EPSPS* gene sequence of GR *A. spinosus* (GenBank: KF569211) was identical with the *EPSPS* gene sequence of GR *A. palmeri* (GenBank: FJ861243), but differed at 29 nucleotides from the *EPSPS* gene sequence of susceptible *A. spinosus* (GenBank: KF569212, KF569213). Nandula et al. (2014) hypothesized that GR in *A. spinosus* was due to pollen-mediated transfer of the GR gene by hybridization with naturally occurring population of GR *A. palmeri*. Fiber-FISH and FISH analyses of GR *A. spinosus* supported this hypothesis and proved that GR *A. spinosus* harbors eccDNA replicon (Figs. 1 and 2), which it most likely acquired from GR *A. palmeri* through interspecific hybridization, as both species are naturally compatible with hybridization (Franssen et al. 2001). The eccDNA replicon in GR *A. spinosus* exhibits structural polymorphism (Fig. 1, A to D) similar to that of GR *A. palmeri* (Koo et al.

Table 1. *EPSPS* gene copy number and eccDNA replicon percent in glyphosate-susceptible (GS) and -resistant (GR) *A. spinosus*, and GR *A. palmeri* plants

Plant	*EPSPS* copy number (No. ± SD)	EccDNA replicon (BAC-FISH)
GS *A. spinosus*	1.0 ± 0.9	0% ($n = 100$)
GR *A. palmeri*	77.3 ± 8.6	>90% (Koo et al. 2018)
GR *A. spinosus*-1*	127.5 ± 4.7	Fiber-FISH
GR *A. spinosus*-2	97.4 ± 10.7	ND
GR *A. spinosus*-4	126.6 ± 7.1	ND
GR *A. spinosus*-5	54.3 ± 2.3	98% ($n = 50$)
GR *A. spinosus*-7	62.4 ± 8.5	ND
GR *A. spinosus*-8	46.3 ± 1.4	ND

The relative *EPSPS*:*β-tubulin* gene copy number was adjusted to 1 for known GS *A. spinosus*, and the copy numbers for GR *A. palmeri* and *A. spinosus* plants shown here are relative to the GS *A. spinosus*. Asterisk indicates the plant that is used in fiber-FISH analysis.
%, mitotic metaphase cell with BAC-FISH signal; ND, not determined.

Table 2. *EPSPS* gene and eccDNA replicon copy number in interspecific F_1 hybrids *A. tuberculatus* (1.0 ± 0.1) × *A. palmeri* (77.3 ± 8.6)

Plant	*EPSPS* copy number (No. ± SD)	EccDNA replicon (BAC-FISH)
GS *A. palmeri*	1.0 ± 0.1	0% (Koo et al. 2018)
W-P#3	3.0 ± 0.8	87% ($n = 30$)
W-P#6	4.4 ± 0.5	93.3% ($n = 30$)

The relative *EPSPS*:*β-tubulin* gene copy number was adjusted to 1 for known GS *A. palmeri*, and the copy numbers for interspecific hybrids shown here are relative to the GS *A. palmeri*.
%, mitotic metaphase cell having BAC-FISH signal.

2018). Both the linear and circular forms of eccDNA replicon occur in the cell. Out of 228 DNA fibers observed, the circular eccDNA replicon was present in 139 DNA fibers, while the linear form of eccDNA replicon was found in 89 DNA fibers. The frequency of linearized eccDNA replicon in GR *A. spinosus* was 34.6%. This frequency may be slightly overestimated, since the breakage of circular eccDNA replicon to linear form due to mechanical force during DNA fiber preparation cannot be ruled out. It is possible that the linear eccDNA replicons were generated from eccDNA replicons associated with replication errors of the eccDNA replicons (Koo et al. 2018). The presence of linear molecules of chloroplast genome is reported due to recombinational events or the random cleavage and fusion of replication intermediates (Lilly et al. 2001). Also, dimeric circular and linear forms of eccDNA replicon with head-to-tail tandem orientation were also observed in GR *A. spinosus* (Fig. 1, C and D).

Further, a controlled hybridization experiment was conducted to demonstrate the transferability of eccDNA replicon during interspecific hybridization. Six F_1 hybrids were developed by hybridizing *A. palmeri* with *A. tuberculatus*. Cytological and molecular analyses of F_1 hybrids corroborated that eccDNA replicon from *A. palmeri* can be transferred by interspecific hybridization (Fig. 2). *Amaranthus palmeri*, *A. tuberculatus*, and *A. spinosus* are members of the family Amaranthaceae and are among the most problematic weeds in the United States. Their floral biology and close genetic relationships favor interspecific hybridization among these species and enable the spread of herbicide resistance (Oliveira et al. 2018). Earlier, we reported the eccDNA replicon-mediated evolution of GR in *A. palmeri* (Koo et al. 2018). This report documents that *A. palmeri*'s eccDNA replicon is capable of pollen-mediated transfer and spread to related species, posing a serious burden on the control of pigweeds in crops.

The hybridization of GR *A. palmeri* (*EPSPS* copy number of 77.3 ± 8.6) with GS *A. tuberculatus* (*EPSPS* copy number of 1) resulted in the true F_1 hybrid W-P#3 with an *EPSPS* copy number of 3.0 ± 0.8 and susceptible to glyphosate (Table 2). The FISH experiments revealed that W-P#3 has a certain percentage of soma cells in its meristematic tissues that harbor a large number of eccDNA replicon signals anchored to the chromosomes (Fig. 2, F and G). The cells with a large number of eccDNA replicon copies can potentially survive glyphosate application, multiply, and produce resistant shoots during the lifetime of this hybrid. Some of the seed borne on the resistant shoots will transmit the GR to the progeny. In cell culture studies exposed to drugs, a similar kind of resistance mediated by episomes (here referred as eccDNA) has been described as an acquired resistance or trait (Stark and Wahl 1984). Our work on *A. palmeri* (Koo et al. 2018) and this report are documented examples of rapid adaptive evolution of a trait in higher organisms mediated by eccDNA. The eccDNAs enable copy number variation in soma cells during the growth of the organism which, under strong selection pressure, may drive rapid adaptive evolution.

Materials and methods

Plant materials

Spiny amaranth (*A. spinosus*) plants were obtained from the field in Lafayette County, Mississippi (34.23450 N, 89.63433 W) and were confirmed to be GR (Nandula et al. 2014). The collected plants were cloned by vegetative propagation in the greenhouse. The interspecific hybrids between *A. tuberculatus* and *A. palmeri* were produced as per the procedure given by Gaines et al. (2012). A total of 6 interspecific F_1 plants were randomly selected for FISH analysis of eccDNA replicons.

Molecular analysis

The quantitative PCR (qPCR) procedure given by Koo et al. (2018) was followed to determine the *EPSPS* copy number in GR *Amaranthus* sp. and their interspecific hybrids. PCR-based species-specific primers developed by Wright et al. (2016) were used for parental species screening and evaluation of F_1 hybrids. The primer information and the PCR set up are given as supplementary information.

Fluorescence in situ hybridization

The procedures for mitotic chromosome preparation, FISH, and fiber-FISH were adapted from Koo et al. (2018). FISH

images were captured with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA) using a cooled CCD camera CoolSNAP HQ2 (Photometrics, Tucson, AZ, USA). Three single-channel (blue, green, and red) images were captured in 8-bit depth black and white and were later superimposed in AxioVision 4.8 software (Carl Zeiss). Assignments for the color of each channel after being superimposed were the same as the fluorochrome color used. The final contrast of the images was processed using Adobe Photoshop CS5 software.

Supplemental data

The following material is available in the online version of this article.

Supplemental Information. The primer sequence information and the PCR set up used in the study.

Acknowledgments

The authors thank W. John Raupp for critical review of the manuscript and Duane Wilson for technical assistance.

Author contributions

D.-H.K., R.S., S.N., and Y.J. performed the experiments; D.-H.K. and B.S.G. wrote the manuscript; V.K.N. confirmed and collected the glyphosate-resistant *A. spinosus*; D.-H.K., B.F., and B.S.G. designed the experiments; D.-H.K., R.S., Y.J., S.N., V.K.N., M.J., B.F., and B.S.G. analyzed the data and helped to draft the final manuscript.

Funding

This research was supported by grants from the Kansas Wheat Commission and the Kansas Crop Improvement Association, Wheat Genetics Resource Center Industry/University Cooperative Research Center National Science Foundation Contract 1822162. This is contribution number 23-216-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506-5502, USA.

Conflict of interest statement. None declared.

References

Cohen S, Houben A, Segal D. Extrachromosomal circular DNA derived from tandemly repeated genomic sequences in plants. Plant J. 2008:**53**(6):1027–1034. https://doi.org/10.1111/j.1365-313X.2007.03394.x

Dillon A, Varanasi VK, Danilova TV, Koo D-H, Nakka S, Peterson DE, Tranel PJ, Friebe B, Gill BS, Jugulam M. Physical mapping of amplified copies of the 5-enolpyruvylshikimate-3-phosphate synthase gene in glyphosate-resistant *Amaranthus tuberculatus*. Plant Physiol. 2017:**173**(2):1226–1234. https://doi.org/10.1104/pp.16.01427

Franssen AS, Skinner DZ, Al-Khatib K, Horak MJ, Kulakow PA. Interspecific hybridization and gene flow of ALS resistance in *Amaranthus* species. Weed Sci. 2001:**49**(5):598–606. https://doi.org/10.1614/0043-1745(2001)049[0598:IHAGFO]2.0.CO;2

Gaines TA, Ward SM, Bukun B, Preston C, Leach JE, Westra P. Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in *Amaranthus* species. Evol Appl. 2012:**5**(1):29–38. https://doi.org/10.1111/j.1752-4571.2011.00204.x

Gaines TA, Zhang W, Wang D, Bukun B, Chisholm ST, Shaner DL, Nissen SJ, Patzoldt WL, Tranel PJ, Culpepper AS. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. Proc Natl Acad Sci USA. 2010:**107**(3):1029–1034. https://doi.org/10.1073/pnas.0906649107

Koo D-H, Molin WT, Saski CA, Jiang J, Putta K, Jugulam M, Friebe B, Gill BS. Extrachromosomal circular DNA-based amplification and transmission of herbicide resistance in crop weed *Amaranthus palmeri*. Proc Natl Acad Sci USA. 2018:**115**(13):3332–3337. https://doi.org/10.1073/pnas.1719354115

Lilly JW, Havey MJ, Jackson SA, Jiang JM. Cytogenomic analyses reveal the structural plasticity of the chloroplast genome in higher plants. Plant Cell. 2001:**13**(2):245–254. https://doi.org/10.1105/tpc.13.2.245

Molin WT, Wright AA, VanGessel MJ, McCloskey WB, Jugulam M, Hoagland RE. Survey of the genomic landscape surrounding the *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene in glyphosate-resistant *Amaranthus palmeri* from geographically distant populations in the USA. Pest Manag Sci. 2018:**74**(5):1109–1117. https://doi.org/10.1002/ps.4659

Molin WT, Yaguchi A, Blenner M, Saski CA. The eccDNA replicon: a heritable, extranuclear vehicle that enables gene amplification and glyphosate resistance in *Amaranthus palmeri*. Plant Cell. 2020:**32**(7):2132–2140. https://doi.org/10.1105/tpc.20.00099

Nandula VK, Wright AA, Bond JA, Ray JD, Eubank TW, Molin WT. *EPSPS* amplification in glyphosate-resistant spiny amaranth (*Amaranthus spinosus*): a case of gene transfer via interspecific hybridization from glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*). Pest Manag Sci. 2014:**70**(12):1902–1909. https://doi.org/10.1002/ps.3754

Oliveira MC, Gaines TA, Patterson EL, Jhala AJ, Irmak S, Amundsen K, Knezevic SZ. Interspecific and intraspecific transference of metabolism-based mesotrione resistance in dioecious weedy *Amaranthus*. Plant J. 2018:**96**(5):1051–1063. https://doi.org/10.1111/tpj.14089

Pennisi E. Circular DNA throws biologists for a loop. Science. 2017:**356**-(6342):996–996. https://doi.org/10.1126/science.356.6342.996

Spier-Camposano H, Molin WT, Saski CA. Sequence characterization of eccDNA content in glyphosate sensitive and resistant palmer amaranth from geographically distant populations. PLoS One. 2022:**17**(9):e0260906. https://doi.org/10.1371/journal.pone.0260906

Stark GR, Wahl GM. Gene amplification. Annu Rev Biochem. 1984:**53**(1):447–491. https://doi.org/10.1146/annurev.bi.53.070184.002311

Steinrücken H, Amrhein N. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem Biophys Res Commun. 1980:**94**(4):1207–1212. https://doi.org/10.1016/0006-291X(80)90547-1

Turner KM, Deshpande V, Beyter D, Koga T, Rusert J, Lee C, Li B, Arden K, Ren B, Nathanson DA. Extrachromosomal oncogene amplification drives tumour evolution and genetic heterogeneity. Nature. 2017:**543**(7643):122–125. https://doi.org/10.1038/nature21356

Wright AA, Molin WT, Nandula VK. Distinguishing between weedy *Amaranthus* species based on intron 1 sequences from the *5-enolpyruvylshikimate-3-phosphate synthase* gene. Pest Manag Sci. 2016:**72**(12):2347–2354. https://doi.org/10.1002/ps.4280