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1 **Running head:** Chromosomal arrangement of amplified *EPSPS* locus

2

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14 **Research area:** Genes, Development and Evolution (for Weed Control Focus Issue)

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15 **Title:**

16 Tandem Amplification of a Chromosomal Segment Harboring *EPSPS* Locus Confers Glyphosate

17 Resistance in *Kochia scoparia*

18

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29

30 **Summary**

31 This research demonstrates tandem arrangement of ten *EPSPS* copies on chromosomes of field-

32 evolved glyphosate-resistant kochia.

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38 **Abstract**

39 Recent rapid evolution and spread of resistance to the most extensively used herbicide,
40 glyphosate, is a major threat to global crop production. Genetic mechanisms by which weeds
41 evolve resistance to herbicides largely determine the level of resistance, and the rate of evolution
42 resistance. In a previous study, we determined that glyphosate resistance in *Kochia scoparia* (L.)
43 Schrad. is due to the amplification of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*)
44 gene, the enzyme target of glyphosate. Here, we investigated the genomic organization of the
45 amplified *EPSPS* copies using fluorescence in situ hybridization (FISH) and extended DNA fiber
46 (Fiber FISH) on *K. scoparia* chromosomes. In both glyphosate-resistant *K. scoparia* populations
47 tested (GR1 and GR2), FISH results displayed a single and prominent hybridization site of the
48 *EPSPS* gene localized on the distal end of one pair of homologous chromosomes compared to a
49 faint hybridization site in susceptible samples (GS1 and GS2). Fiber FISH displayed ten copies
50 of the *EPSPS* gene (~5 kb), arranged in tandem configuration of ~40 to 70 kb apart with one
51 copy in an inverted orientation (total length ~511 kb) in GR2. In agreement with FISH results,
52 segregation of *EPSPS* copies followed single locus inheritance in GR1 population. This is the
53 first report of tandem target gene amplification conferring field-evolved herbicide resistance in
54 weed populations.

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55 Glyphosate (N-(phosphonomethyl) glycine) is the most widely used agricultural pesticide
56 globally (Duke and Powles, 2008). Originally, being a non-selective herbicide, its use was
57 limited to vegetation management in non-crop areas; however, introduction of glyphosate-
58 resistant (GR) crops in the late 1990's, coupled with their exceptional adoption, led to
59 accelerated use totaling ~128 million ha worldwide in 2012 (James, 2012). GR crop technology
60 has made a significant contribution to global agriculture and the environment as it not only
61 increased farm income by $32.2 billion (Brookes and Barfoot, 2013) but also moderated the
62 negative environmental impacts of mechanical weed management practices (Gardner and
63 Nelson, 2008; Bonny, 2011). Glyphosate offers a simple, effective and economic weed
64 management option in GR crops. In addition, it provides immense value in no-till crop
65 production systems by enabling soil and moisture conservation. However, due to intensive
66 glyphosate selection pressure, several weed populations globally have evolved resistance through
67 a variety of mechanisms. Globally, herbicide resistance, in particular the recent proliferation of
68 glyphosate resistance in weed species is a major crop protection threat; nearly two dozen GR
69 weed species have been reported in the last 15 years (Heap, 2014).
70 Glyphosate, an aminophosphonic analogue of the natural amino acid glycine, non-
71 selectively inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in plants preventing
72 the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Steinrücken
73 and Amrhein, 1980), resulting in the death of glyphosate-sensitive individuals. In plants, EPSPS
74 is one of the key enzymes in the shikimate pathway (Herrmann and Weaver, 1999) and
75 glyphosate inhibits EPSPS by binding to EPSPS- shikimate-3-phosphate (S3P) binary complex
76 forming an EPSPS-S3P-glyphosate complex (Alibhai and Stalling, 2001). Bradshaw et al.
77 (1997) hypothesized against the likelihood of weeds evolving resistance to glyphosate, primarily

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78 because of its complex biochemical interactions in the shikimate pathway, and also due to the
79 absence of known glyphosate metabolism in plants. Nonetheless, several cases of glyphosate
80 resistance, as a result of difference in glyphosate translocation (Preston and Wakelin, 2008) or
81 mutations in the *EPSPS*, were confirmed (Baerson et al., 2002). More importantly,
82 duplication/amplification of the *EPSPS* appears to be the basis for glyphosate resistance in
83 several weeds (Sammons and Gaines, 2014). Here, we use 'duplication' to refer to the formation
84 of first repetition of a chromosomal segment and 'amplification' to refer to increase in number of
85 the repetitions (more than two repetitions of a chromosomal segment) under positive selection.
86 The first case of *EPSPS* amplification as a basis for glyphosate resistance was reported in an
87 *Amaranthus palmeri* population from Georgia (Gaines et al., 2010). In this *A. palmeri*
88 population, there is a massive increase (>100-fold relative to glyphosate-susceptible plants) in
89 *EPSPS* copies and these copies are dispersed throughout the genome (Gaines et al., 2010).
90 Field-evolved GR *K. scoparia* (L.) Schrad. populations were first reported in western KS,
91 USA in 2007 (Heap, 2014). We previously determined that evolution of GR-populations of *K.*
92 *scoparia* (L.) Schrad. in the US Great Plains is also due to amplification of the *EPSPS* (Wiersma,
93 et al., unpublished). Unlike in GR *A. palmeri*, we found relative *EPSPS:* acetolactate synthase
94 (*ALS*) copies ranging from 3 to 9 in GR *K. scoparia* populations. While it quickly became
95 widespread in the region (Wiersma, et al., unpublished), its presence was reported in another five
96 Great Plains states by 2013 (Heap, 2014). GR *K. scoparia* populations we tested were 3- to 11-
97 times resistant (population level) to glyphosate compared to a glyphosate susceptible population
98 (Godar, 2014) and *EPSPS* expression positively correlated with genomic *EPSPS* copy number
99 (Wiersma, et al., unpublished). Here, we reveal the genomic organization of the amplified

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100 *EPSPS* copies in two GR *K. scoparia* populations, an alternative mechanism of gene
101 amplification than reported in GR *A. palmeri.*
102
103 **RESULTS**
104 **Chromosomal Location of the Amplified *EPSPS* Copies.** To investigate the location of
105 amplified *EPSPS* gene copies on GR *K. scoparia* chromosomes, we used fluorescence in situ
106 hybridization (FISH). Analysis of FISH showed a marked increase in *EPSPS* signal in GR *K.*
107 *scoparia* plants, relative to glyphosate-susceptible plants (Fig. 1). In GS1 and GR1 *K. scoparia*,
108 three chromosome pairs with nucleolus organizer regions (NORs) were detected; one of which,
109 with a minor NOR signal, had the *EPSPS* gene on the distal end (Fig. 1, A and B). On metaphase
110 spreads, the *EPSPS* probe detected much brighter signal on GR1 chromosome pair relative to the
111 signal on GS1 chromosome pair (Fig. 1, A and B). Similar *EPSPS* signals were observed on
112 metaphase chromosomes of GS2 and GR2 plants (Fig. 1, C and D). On prometaphase
113 chromosomes and interphase nuclei, only faint *EPSPS* signal was seen on each chromatid
114 (Supplemental Fig. S1, A and C) on GS1 samples whereas 5-7 partially overlapping signals of
115 the *EPSPS* probe could be distinguished at this location on GR1 samples (Supplemental Fig. S1,
116 C and D).
117 **High Resolution Mapping of the *EPSPS* Cluster.** To further explore the arrangement of
118 *EPSPS* gene copies, we first performed FISH on stretched DNA fiber (Fiber FISH) on a GS2 and
119 GR2 plants using one-color followed by two-color probes. High resolution images of Fiber FISH
120 results showed only one *EPSPS* copy in GS2 plants (Supplemental Fig. S2A). In agreement with
121 the brighter probe hybridization signal in FISH results (Fig. 1D), we observed tandemly
122 configured ten copies of *EPSPS* gene on a single DNA fiber of GR2 plants (Supplemental Fig.

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123 S2B). On metaphase chromosomes of GR2 plant, the *EPSPS* probe hybridized to the same
124 location giving comparable signal intensity (Fig. 2, A-C) as previously observed in Fig. 1C. The
125 two-color probes on a single DNA fiber detected ten *EPSPS* copies, one with an inverted *EPSPS*
126 sequence (Figs. 2, D-F). The total length of the amplified region (measured on seven individual
127 DNA fibers) is approximately 511 ± 26 kb. The *EPSPS* copies are located approximately 40 to
128 70 kb apart on a GR2 *K. scoparia* chromosome (Fig. 2G).

129 **Inheritance of Glyphosate Resistance.** We investigated the inheritance of glyphosate resistance
130 in *K. scoparia* using a classical genetic approach. We selected non-segregating GS1 and GR1
131 lines (see materials and methods section) as parents to generate F_1 progeny. F_1 seed was
132 successfully generated from reciprocal crosses. F_2 seed was produced by self-pollinating F_1
133 plants. In response to a field use rate of glyphosate (868 kg ae ha^{-1}) application, the GS plants
134 showed stunted growth and eventually died, while GR plants exhibited little or no injury and
135 continued to grow normally (Fig. 3A). As expected for a nuclear inherited *EPSPS* gene, F_1 plants
136 derived from either GR x GS or GS x GR crosses survived 868 g ae ha^{-1} glyphosate application
137 (Fig. 3A). A total of 115 F_2 plants were evaluated for glyphosate resistance with the same dose
138 (868 g ae ha^{-1}) of glyphosate. F_2 progeny segregated as 85 GR: 30 GS (3 GR: 1 GS with χ^2 =
139 0.072), fitting a single locus inheritance.

140 **Segregation of *EPSPS* Copies in F_1 and F_2 Progeny.** The *EPSPS* copies in parental plants (n =
141 2, GS1; n = 4, GR1), F_1 (n = 6) and F_2 progeny (n = 50) was determined using quantitative PCR
142 on genomic DNA. GR1 parental plants possessed 9 to 11 copies relative to GS1 plants (Fig. 3B).
143 F_1 progeny had ~ 5 to 7 *EPSPS* copies (Fig. 3B). In F_2 progeny, the copy number ranged from 1
144 to 13 at a frequency of 24 and 76% with 1 and 4-13 *EPSPS* copies, respectively. The *EPSPS*

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145 copy number observed in our genetic populations is in agreement with the inheritance of
146 glyphosate resistance.

147 We also measured the amount of shikimate accumulated in glyphosate-treated leaf discs
148 to estimate level of glyphosate resistance. Shikimate accumulation results in plants when *EPSPS*
149 is inhibited by glyphosate (Amrhein et al., 1980; Herrmann and Weaver, 1999), thus the degree
150 of shikimate accumulation may be used as an indirect measure of plant's sensitivity to
151 glyphosate. In this case, if there are higher *EPSPS* copies, less shikimate accumulation was
152 expected. GR parents accumulated less shikimate in leaf discs compared to GS parents (Fig. 3B).
153 F_1 plants accumulated drastically more shikimate than GS parents ($P < 0.001$) and less shikimate
154 than GR parents ($P = 0.064$). Overall, shikimate accumulation correlated with the *EPSPS* copy
155 number ($r = -0.841$).

156 ***K. scoparia* Populations Show Increase in *EPSPS* Copies and Level of Resistance Over**
157 **Years:** We have been collecting field populations of *K. scoparia* over several years to test
158 whether in response to glyphosate application (selection event), there is an increase in *EPSPS*
159 copy number due to recombination and selection at *EPSPS* locus. We estimated the *EPSPS*
160 copies and level of glyphosate resistance in GR *K. scoparia* plants from populations that were
161 collected in KS in 2007, 2010 and 2012. The results suggest that the GR *K. scoparia* plants
162 collected in 2007 possessed an average of 9 *EPSPS* copies, while plants from 2010 and 2012
163 collection, had up to 12 and 16 copies, respectively (Fig. 4). Furthermore, GR *K. scoparia* plants
164 with 9 and 12 copies withstood 1736 g ae ha^{-1}, but did not survive 3472 g ae ha^{-1} glyphosate.
165 However, plants from 2012 collection survived up to 5208 g ae ha^{-1} glyphosate rate (Fig. 4),
166 implying a progression in *EPSPS* copies and level of glyphosate resistance from 2007 to 2012.

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167 These data indicate that, with glyphosate selection events, *K. scoparia* populations with

168 increased *EPSPS* copies, likely arose due to unequal crossing over, may have been favored.

169

170 **DISCUSSION**

171 This study reports the first case of tandem amplification of a target site as a mechanism of

172 naturally evolving resistance to herbicides in plants. Massive amplification of the *EPSPS* gene

173 randomly dispersed throughout the genome (Gaines et al., 2010), likely mediated by transposable

174 elements (Gaines et al., 2013), has been recently reported in GR *A. palmeri*. Tandem

175 amplifications of genes that metabolize insecticides have been reported in organophosphate-

176 resistant populations of *Culex* mosquitoes (Field and Devonshire, 1997) and *Myzus persicae*

177 (Paton et al., 2000). Our results demonstrate the tandem amplification of a target gene itself as a

178 basis for mechanism of herbicide resistance. An intriguing question concerning the resistance

179 mechanism to glyphosate is whether *EPSPS* copy number increased in response to a positive

180 selection or whether rare plants with multiple copies existed prior to selection. To date, at least

181 one example of preexistence of the multi-copy target gene has been reported, which results in

182 resistance to kinase inhibitors used in lung cancer therapy (Turke et al., 2009).

183 In nature, gene duplication is common phenomenon and is a precursor for genetic

184 diversity (Wagner et al., 2007). The significance of gene duplication has been comprehensively

185 reviewed (Van de Peer, 2004; Bailey and Eichler, 2006; Conant and Wolfe, 2008; Ponting,

186 2008). Some duplicated genes confer an immediate adaptive advantage (Perry et al., 2007) and

187 provide a substrate for further amplification under selection, i.e. adaptive amplification. One of

188 the common mechanisms of such amplification is unequal crossing over that takes place between

189 homologues or sister chromatids within the amplified region (Bindbergen, 2011). Role of

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190 unequal meiotic recombination in the formation of many disease resistance gene clusters in crop
191 plants has been documented (Van der Hoorn et al., 2001; Nagy and Bennetzen, 2008; Luo et al.,
192 2011). Continuous variation in *EPSPS* copy number, and a positive correlation between *EPSPS*
193 expression and the copy number that we have seen (Wiersma, et al., unpublished), suggests that
194 the *EPSPS* copy number in *K. scoparia* plants increases through an adaptive process.
195 Furthermore, hybridization of *EPSPS* probes at distal ends of homologous chromosomes of *K.*
196 *scoparia* (Fig. 1) also suggests that increase in *EPSPS* copies in GR *K. scoparia* may have
197 occurred as a result of unequal cross over, as the gene duplication via unequal cross over most
198 likely occurs at telomere region of chromosomes (Royle et al., 1988; Amarger et al., 1998; Ames
199 et al., 2008).
200 Here, we illustrate a model for *EPSPS* amplification via unequal crossover in response to
201 glyphosate selection in *K. scoparia* (Fig. 5). Survival of the plant that inherits duplicated *EPSPS*
202 copy (heterozygous for the duplicated copy) from the first duplication event is critical for
203 evolution of resistance to glyphosate as such an individual will have only a slightly elevated
204 advantage under glyphosate selection. Factors including lower-than normal use rates of
205 glyphosate, environmental stress (drought and high temperatures), and incomplete spray
206 coverage may result in its survival, thereby, allowing it to establish and reproduce one-fourth of
207 the progeny homozygous for the duplicated copies. Sequence homology between the duplicated
208 segments provides a substrate for unequal crossover to happen leading to *EPSPS* copy number
209 gain and loss in next generation progeny. Under continuous selection, plants with higher *EPSPS*
210 copies will be selected until the return to additional gain in the copy number reaches plateau.
211 Rate of copy number increase will depend on the interplay between number of repetitions of
212 sequence homology (copy number) and the return to the gain in additional copy number.

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213 Models for predicting spread of herbicide resistance in weeds largely rely on underlying
214 genetic mechanisms (single vs polygenic, maternal vs. nuclear-inherited) and levels of resistance
215 conferred in different states of zygocity. The single locus inheritance of the *EPSPS* copies or
216 glyphosate resistance we observed in our classical genetic population is in conformity with
217 tandem arrangement of *EPSPS* copies. In the context of gene amplification, cytogenetic
218 arrangement of the amplified gene (tandem vs. dispersed), their stability as well as the magnitude
219 of selection pressure determine dynamics of the resistance locus (loci) not only at the population
220 but also at the individual level unlike in classical genetic models. *EPSPS* transcript analysis
221 showed that there were no polymorphism in *EPSPS* transcript sequences and no *EPSPS* splice
222 variants were detected in GR *K. scoparia* (Wiersma, et al., unpublished). Although initially most
223 of *EPSPS* copies, if not all, will have a complete set of functional motifs and be functionally
224 indistinguishable from the original copy, over time amplified sequences may diverge and code
225 for new functions. Whether increase in *EPSPS* gene copies affects fitness of the plants in the
226 absence of selection is unknown, however, a many-fold increase of *EPSPS* genes in GR *A.*
227 *palmeri* does not accrue a measurable fitness cost (Giacomini et al., 2014; Vila-Aiub et al.,
228 2014). These two factors along with the magnitude of selection pressure will shape the way for
229 additional increases in the *EPSPS* copy number.

230 Our results revealed the tandem genomic organization of amplified *EPSPS* copies in two
231 GR *K. scoparia* populations. Nonetheless, the significance of the inverted *EPSPS* copy in the
232 evolution of glyphosate resistance remains unknown. Although, our previous study shows
233 similar levels of *EPSPS* amplification in other GR *K. scoparia* populations from the US Great
234 Plains, whether similar amplification patterns exist in those populations remains to be seen.
235 Further investigation of flanking sequences including breakpoints will shed additional light on

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236 the mechanism of such possibly recurrent rearrangement events that resulted in evolution of

237 glyphosate resistance in *K. scoparia* populations.

238

239 MATERIALS AND METHODS

240 Plant materials

241 Plants from two GS K. scoparia populations; GS1 and GS2, and two GR *K. scoparia*

242 populations; GR1 and GR2 all from Kansas, USA were used in this study.

243

244 FISH procedure

245 Somatic chromosome preparations (using the drop technique), direct probe labeling (by nick

246 translation), and the FISH procedure on GS1 and GR2 plants were performed as described

247 previously (Kato et al., 2004; Kato et al., 2006) with minor modifications. Root tips were

248 collected from young plants and treated in a nitrous oxide gas chamber for 1 hr 30 min, fixed on

249 ice in cold 90% acetic acid for 10 min, washed and stored in 70% ethanol at −20°C. For slide

250 preparation, roots were washed in tap water for 10 min and then in KCl buffer for 5 min (75 mM

251 KCl, 7.5 mM EDTA, pH 4); 7 meristems (0.5 to1 mm long) were placed in 20 μL of 4%

252 cellulase Onozuka R-10 (Yakult, Japan, Tokyo cat # 201069), 1% pectolyase Y23 (Karlan cat #

253 8006) in KCl buffer, and incubated for 43 min at 37°C. Digested meristems were washed for 5

254 min in ice-cold Tris–EDTA buffer, pH 7.6, then three additional washes in 100% ethanol.

255 Meristems were dispersed with a needle in 20 μL of ice-cold acetic acid:methanol mix (9:1) and

256 immediately dropped on to 3 pre-cleaned glass slides placed in a humid chamber. Dried

257 preparations were UV cross-linked, soaked in methacarn solution (methanol: chlorophorm :

258 glacial acetic acid 6:3:1) for 1 min, dried and used for hybridization on the same day. For

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259 labeling the nucleolus organizing region (NOR) rRNA loci, clone pTa71, containing a 9-kb
260 insertion with 18S, 5.8S, and 26S rRNA wheat genes and intergenic spacers (Gerlach and
261 Bedbrook, 1979) was used as a probe. Five μL of probe mixture contained 200 *n*g of each
262 EPSPS gene PCR product labeled with Texas red-5-dCTP and 160 ng of pTa71 labeled with
263 Fluorescein-12-dUTP (PerkinElmer, cat # NEL413001EA and NEL426001EA). The mixture of
264 probes and the slide preparation were denatured at 100°C separately. The rest of the FISH
265 procedure and washes were performed by using the method described by Kato et al. (2006).
266 The FISH on somatic metaphase chromosome of GS2 and GR2 was performed using a
267 procedure as described by Koo et al. (2010). Biotin- and digoxigenin-labeled probes were
268 detected with Alexa Fluor 488 streptavidin antibody (Invitrogen, Carlsbad, CA) and rhodamine-
269 conjugated anti-digoxigenin antibody (Roche Diagnostics USA, Indianapolis, IN), respectively.
270 In both FISH experiments, chromosome preparations were mounted and counterstained
271 with 4', 6-diamidino-2-phenylindole solution (DAPI) in Vectashield (Vector Laboratories, cat #
272 H-1200, H-1300). FISH images were captured with a Zeiss Axioplan 2 microscope using a
273 cooled charge-coupled device camera CoolSNAP HQ2 (Photometrics) and AxioVision 4.8
274 software (Zeiss). The final contrast of the images was processed using Adobe Photoshop CS5
275 software (Adobe Systems Incorporated, San Jose, CA, USA).
276
277 **EPSPS FISH probe preparation**
278 Sequences of *K. scoparia EPSPS* mRNA (14) and the *A. palmeri EPSPS* gene (GenBank
279 Accession JX56456) were used to develop the PCR primers (Supplemental Table S1;
280 Supplemental Fig. S3). The *EPSPS* gene was amplified using PSI *K. scoparia* genomic DNA as
281 a template isolated with Qiagen DNeasy Plant Mini kit (cat. # 69104). The PCR reaction

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282 included JumpStart REDTaq ReadyMix (Sigma, Cat. P0982), 0.4 μM of each primer and 0.5 to 4

283 ng μL^{-1} of template DNA. PCR cycles consisted of 96°C, 5 min -initial denaturation, 35 cycles:

284 96°C - 30 sec, 57°C - 30 sec, 72°C - 4 min, and final extension of 15 min. PCR products were

285 cut and eluted from agarose gel with Qiagen Gel Extraction kit (Cat. # 28706) and reamplified

286 using the same primers. PCR products were purified with Invitrogen PCR Purification kit (Cat. #

287 K3100-01) and verified by sequencing (Genewiz). The sequence of amplified part of *K. scoparia*

288 *EPSPS* gene was submitted to NCBI GenBank database with accession number KJ374721. Three

289 PCR products were tested separately by FISH and products 1 and 3 that showed no background

290 staining on *K. scoparia* chromosomes were used as a pooled FISH probe.

291

292 **Fiber-FISH procedure**

293 Young leaf tissues were collected from fast growing plants of GS2 and GR2. Nuclei isolation,

294 DNA fiber preparation, and Fiber FISH were performed following published protocols (Jackson

295 et al., 1998; Koo et al., 2011). Fiber FISH images were captured and processed as previously

296 described in FISH procedure. The cytological measurements of the fiber-FISH signals were

297 converted into kilobases using a 3.0 kb μm^{-1} conversion rate.

298

299 **EPSPS Fiber-FISH probe preparation**

300 Sequences of *A. palmeri EPSPS* gene (GenBank Accession JX564536) were used to develop the

301 PCR primers (Supplemental Fig. S4) for cloning *EPSPS* gene from *K. scoparia*. The probes were

302 amplified with primers (Supplemental Table S1) using GS2 plant genomic DNA as a template.

303 PCR product was cloned in 2.1-TOPO® TA vector and the clones were labeled with either

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304 biotin-16-UTP or digoxigenin-11-dUTP (Roche Diagnostics, Indianapolis, IN) using a standard
305 nick translation reaction.
306
307 **Creation of Genetic Populations and Their Phenotyping**
308 Plants from *K. scoparia* populations GS1 and GR1 were grown in a greenhouse. Before
309 flowering, the whole plant was covered with plastic bread bags (33 cm x 60 cm) with micro-
310 perforations for self-pollination. Upon plant maturity, seed was harvested separately from
311 individual plants. A subset of selfed progeny seeds were planted and about 40 plants (8 to10 cm
312 tall) from each line were treated with glyphosate (Roundup WeatherMAX, Monsanto Company,
313 St. Louis, MO) at the rate of 868 g ae ha^{-1} using a chamber sprayer calibrated to deliver 187 L ha^{-}
314 ^{1}. None of the plants from GS1 population survived the glyphosate treatment, hence, identified as
315 a GS line. All plants from GR1 population survived the glyphosate treatment, therefore,
316 considered for crossing as a GR line. Reciprocal crosses of GS1 and GR1 (R x S represents a GR
317 female pollinated with GS pollen and vice versa for S x R) plants were performed as follows. *K.*
318 *scoparia* bears protogynous flowers with stigma being receptive for one week before anthesis of
319 the same flower. Therefore, prior to stigma emergence, all the leaves and apical meristems were
320 removed from few randomly selected branches of GR or GS plants, and covered with Lawson
321 217 pollination bags. After stigma emergence, using a sterile forceps, pollen from the dehisced
322 anthers of GR or GS plants (chosen as male parents) was transferred separately onto the stigmas
323 of the maternal flowers. Immediately after pollination, the flowers were covered with the same
324 pollination bags for 10 days. Mature F_1 seed were harvested separately from reciprocal crosses.
325 F_1 plants were treated with glyphosate as described above and all F_1 plants from both reciprocal
326 crosses survived 868 g ae ha^{-1} rate of glyphosate. Randomly selected F_1 plants were transplanted

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327 into larger pots, self-pollinated (using the plastic bread bags with micro-perforations as
328 mentioned earlier) and F_2 seed was harvested separately. F_2 plants were treated with 868 g ae ha^{-1}
329 rate of glyphosate as described above.

330

331 **Shikimate Accumulation Assay**

332 An in vivo measure of shikimate accumulation was determined on parental, F_1 and F_2 progeny
333 following the procedure developed by Shaner et al. (2005). Six 4-mm leaf disks were collected
334 from a fully-expanded young leaf of a single plant when 8 to10 cm tall. Disks were placed in a
335 96-well plate with one disk per well containing 100 μM glyphosate solution. Leaf discs were
336 incubated for 16 hr under continuous light (μmol 250 $m^{-2}s^{-1}$). A shikimate standard curve was
337 used to calculate the shikimate accumulation in ng shikimate μL^{-1}. The experiment was done in
338 triplicate and repeated.

339

340 **Quantitative PCR**

341 *EPSPS* gene copy number was determined in parental, F_1 and F_2 plants. Leaf tissue samples were
342 collected in 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen and stored
343 at -20°C. DNA was extracted using the Qiagen DNEasy Plant Mini Kit (Qiagen Inc., Valencia,
344 CA) and quantified using a NanoDrop spectrophotometer. Relative *EPSPS* gene copy number
345 was determined by quantitative PCR (qPCR) on genomic DNA (gDNA) using acetolactate
346 synthase (*ALS*) as a reference gene. *EPSPS* forward and reverse primers were 5’
347 GGCCAAAAGGGCAATCGTGGAG 3’ and 5’ CATTGCCGTTCCCGCGTTTCC 3’,
348 respectively. ALS forward and reverse primers were 5’ ATGCAGACAATGTTGGATAC 3’ and
349 5’ TCAACCATCGATACGAACAT 3’, respectively. These primers produce products of 102

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350 and 159 bp for *EPSPS* and *ALS*, respectively. qPCR was performed using 96-well plates with
351 each well containing a master mix comprised of: 10 μL of iQ™ SYBR® Green Super Mix
352 (BioRad Inc., Hercules, CA), 1 μL of each corresponding forward and reverse primer, 16 ng of
353 gDNA, and 4 μL of diH_2O. Each reaction was done in triplicate and was repeated. PCR cycle
354 parameters were set for 95°C for 3 min, denaturing at 95°C for 10 sec, annealing and extension
355 at 60°C for 30 sec and the denaturing/annealing steps repeated for 39 cycles. Relative
356 quantification of *EPSPS* was calculated as $\Delta Ct = Ct_{ALS} - Ct_{EPSPS}$ and *EPSPS* copy number was
357 expressed as $2^{\Delta Ct}$. The copy number was averaged across replications and the standard deviation
358 was calculated for each plant sample.
359

360 ***EPSPS* Copies and Level of Resistance in *K. scoparia* Populations Collected Over Years**
361 *EPSPS* copies and level of resistance to glyphosate was determined in *K. scoparia* populations
362 collected over years. Seed of GR *K. scoparia* were collected from fields in 2007, 2010 and 2012.
363 Levels of resistance to glyphosate on 20 to 32 plants from each collection was determined by
364 shikimate accumulation assay and/or treating whole plants with varying rates of glyphosate (0 to
365 5208 g ae ha^{-1}) as previously described. *EPSPS* copy number of at least six plants from each
366 collection that showed highest levels of resistance were determined using qPCR as previously
367 described.
368

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371

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372 **Author contributions:** M.J., K.N., A.S.G., D.K., T.D., S.S., and B.S.G. designed research;
373 K.N., A.S.G., D.K., T.D., and V.K.V performed research; M.J., K.N., A.S.G., D.K., T.D., B.F.,
374 S.S., V.K., A.W., P.W., P.W.S., and B.S.G. analyzed data; and J. M. and A.S.G. wrote this
375 paper.

376 The authors declare no conflict of interest.

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480 **Figure Legends**

481 **Figure 1.** FISH mapping of *EPSPS* gene on chromosome of glyphosate-susceptible (GS) (A and
482 C) and glyphosate-resistant (GR) (B and D) *Kochia scoparia*: Three NOR sites on somatic
483 metaphase chromosome pairs of GS1; one of which with a minor NOR signal showing faint
484 *EPSPS* signal on the distal end (A). Somatic metaphase spreads of GR1 showing bright *EPSPS*
485 signal on the same chromosome pair with a minor NOR site (B).FISH mapping of *EPSPS* gene
486 on somatic metaphase chromosomes of GS2 (C) and GR2 (D) *Kochia scoparia*. Arrows point
487 chromosomes with *EPSPS* signal. Bar, 10 µm.

488 **Figure 2.** High resolution Fiber Fish mapping of *EPSPS* of glyphosate-resistant (GR2) *Kochia*
489 *scoparia.* Metaphase chromosome spread in two-color FISH showing *EPSPS* copies clustered at
490 the distal end of homologous chromosomes (A, B, and C). Hybridization of two colored (red and
491 green) *EPSPS* probes on five different chromosome fibers (D and E). F and G represent
492 orientation of *EPSPS* copies and estimated distance between two adjacent *EPSPS* copies based
493 on figures D and E. Bar, 10 µm. Red signal (~ 1.9 kb) and green signal (~ 2.5 kb) encompass the
494 entire length of *EPSPS* in *Kochia scoparia*. Measurement of cluster of *EPSPS* genes was 511.8 ±
495 26.0 kb (n = 7) in length.

496 **Figure 3.** Response to glyphosate-susceptible (GS), F_1 plant (F_1), and glyphosate-resistant (GR)
497 to 868 g ae ha^{-1} rate of glyphosate (A). Relationship between *EPSPS* genomic copy number and
498 shikimate accumulation in 2 glyphosate-susceptible (filled circles) (B), 4 glyphosate-resistant
499 (filled squares) and 6 F_1 plants (filled diamonds) of *Kochia scoparia*. *EPSPS* copy number and
500 accumulation of shikimate were determined as described in *Materials and Methods*.

501 **Figure 4.** *EPSPS* genomic copies and level of glyphosate resistance in *Kochia scoparia* plants
502 collected in 2007, 2010 and 2012.

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503 **Figure 5.** Illustration of a model suggesting *EPSPS* amplifications via unequal crossover in

504 response to glyphosate selection and evolution of glyphosate resistance in *Kochia scoparia.*

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513 **Supplemental Table S1.** Primers used to develop *EPSPS* gene FISH and Fiber FISH probes.

	Primer	Primer (5'-3')	PCR amplicon length (bp)
1	P1F	GGCTCAAGCTACCACCTTTAACAA	1,907
	P1R	GCAGGAAATAGACCGCCACA	
2	P2F	CTAAGAACTCTTGGGCTCAACGT	1,207
	P2R	CCCTGGAAGACCTCCTTTAGCA	
3	P3F	CAAATTGTCCTCCTGTAAGAGTAA	~ 1,730
	P3R	CTTCACTCTCCAGCTAGCAAC	
4	P4F	CCAAAACCCAGTTACCCAAA	~1,900
	P4R	ACAGACCACCACAACCCTCT	
5	P5F	CTTCCAGGGGGCAAGGTAA	~2,500
	P5R	CAAGGATAGTGACGGGAACA	

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515 **Supplemental Figure Legend**

516

517 **Supplemental Figure S1.** FISH mapping of *EPSPS* gene on chromosome of glyphosate-
518 susceptible GS1 and glyphosate-resistant GR1 *Kochia scoparia*. (A) Prometaphase chromosomes
519 and (C) interphase nuclei of GS1 showing only one EPSPS signal on each chromatid. (B)
520 Prometaphase chromosomes and (D) interphase nuclei of GR1 distinguishing 5-7 partially
521 overlapping signals of the EPSPS probe.

522 **Supplemental Figure S2.** High resolution of Fiber FISH results showing chromosomal
523 distribution *EPSPS* copies in GS and GR *Kochia scoparia* plants. (A) single and (B) ten copies
524 of *EPSPS* gene in GS2 and GR2 plants, respectively. Green signals (~5 kb) encompass the entire
525 *EPSPS* gene in *Kochia scoparia.* Bar, 10 μm.

526 **Supplemental Figure S3.** FISH probe design. Part of *Kochia scoparia EPSPS* sequence (total
527 length = 4653 bp) used for FISH probe production and sequencing. Sequences of mRNA are
528 shown as green rectangles. Primer positions in mRNA are shown on top. This probe was used in
529 FISH procedure in GS1 and GR1 plants.

530 **Supplemental Figure S4.** *EPSPS* structure of *Amaranthus palmeri* and PCR primer positions for
531 *Kochia scoparia EPSPS* cloning for FISH and Fiber FISH probe preparation. This probe was
532 used in FISH and Fiber FISH procedure in GS2 and GR2 plants. PCR products, ~1,900 bp
533 (primers designed from Exon 1 and Exon 2 of *Amaranthus palmeri*) and ~2,500 bp (Exon 4 and
534 Exon 8) were cloned and used as FISH probes. All primer sequences are shown in Supplemental
535 Table S1.

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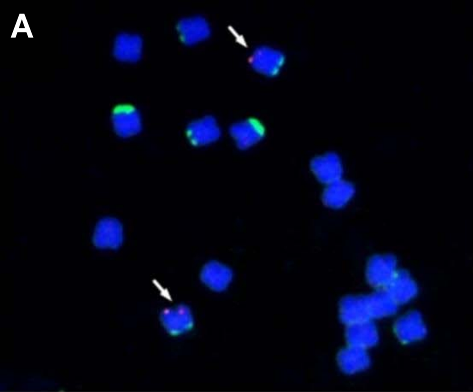
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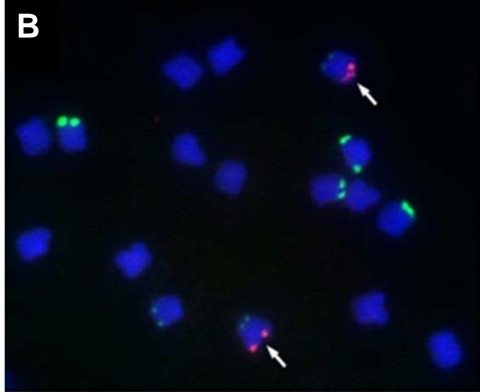
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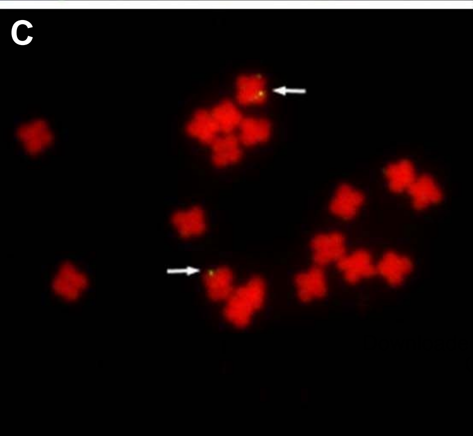
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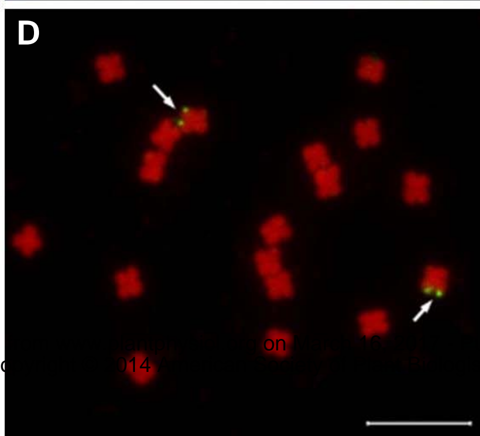
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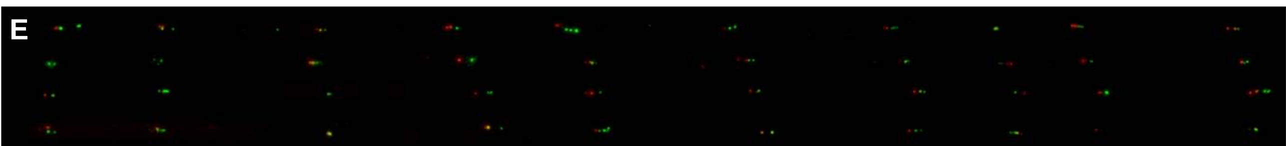

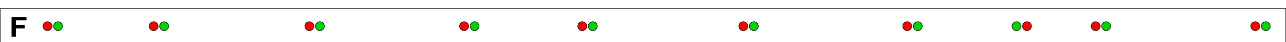

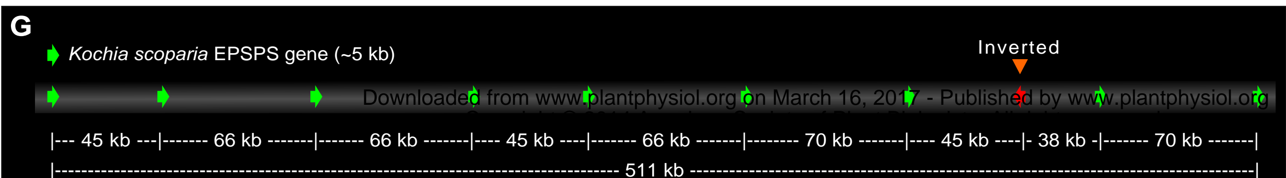

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A

GS

F_1

GR

B

Shikimate accumulation (ηg µL^{-1})

30
25
20
15
10
5
0

0 2 4 6 8 10 12

EPSPS copy number

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2007 collection
9
EPSPS copies

------- 2010 collection -------
12
EPSPS copies

2012 collection
16
EPSPS copies

217	1736	1736	3472	5208
g ae ha^{-1}	g ae ha^{-1}	g ae ha^{-1}	g ae ha^{-1}	g ae ha^{-1}

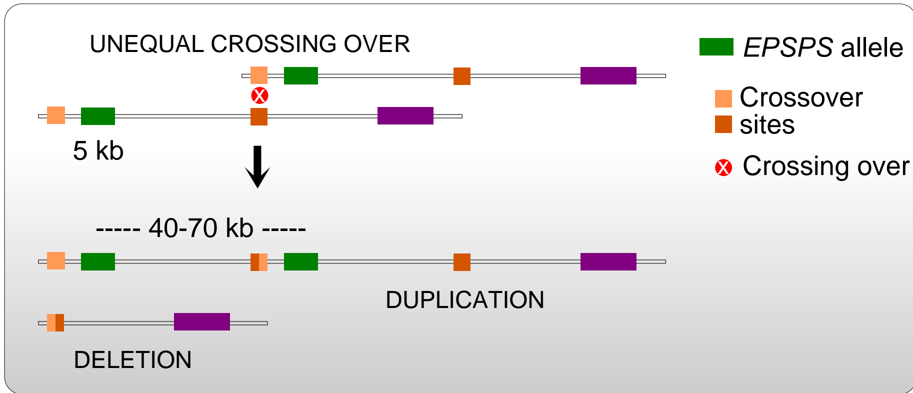
SUSCEPTIBLE

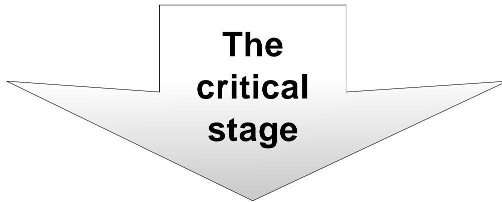
------------------------------------- RESISTANT -------------------------------------

INITIAL DUPLICATION OF *EPSPS* GENE

via UNEQUAL CROSSING OVER

THE FIRST EVENT





ADAPTIVE AMPLIFICATION

UNEQUAL CROSSING OVER

DUPLICATION

DELETION

GLYPHOSATE SELECTION

GLYPHOSATE SELECTION

GLYPHOSATE SELECTION

Increasing *EPSPS* copy number

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