Tandem Amplification of a Chromosomal Segment Harboring *5-Enolpyruvylshikimate-3-Phosphate Synthase* Locus Confers Glyphosate Resistance in *Kochia scoparia*[1][W][OPEN]

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Recent rapid evolution and spread of resistance to the most extensively used herbicide, glyphosate, is a major threat to global crop production. Genetic mechanisms by which weeds evolve resistance to herbicides largely determine the level of resistance and the rate of evolution of resistance. In a previous study, we determined that glyphosate resistance in *Kochia scoparia* is due to the amplification of the *5-Enolpyruvylshikimate-3-Phosphate Synthase* (*EPSPS*) gene, the enzyme target of glyphosate. Here, we investigated the genomic organization of the amplified *EPSPS* copies using fluorescence in situ hybridization (FISH) and extended DNA fiber (Fiber FISH) on *K. scoparia* chromosomes. In both glyphosate-resistant *K. scoparia* populations tested (GR1 and GR2), FISH results displayed a single and prominent hybridization site of the *EPSPS* gene localized on the distal end of one pair of homologous metaphase chromosomes compared with a faint hybridization site in glyphosate-susceptible samples (GS1 and GS2). Fiber FISH displayed 10 copies of the *EPSPS* gene (approximately 5 kb) arranged in tandem configuration approximately 40 to 70 kb apart, with one copy in an inverted orientation in GR2. In agreement with FISH results, segregation of *EPSPS* copies followed single-locus inheritance in GR1 population. This is the first report of tandem target gene amplification conferring field-evolved herbicide resistance in weed populations.

Glyphosate [*N*-(phosphonomethyl) Gly] is the most widely used agricultural pesticide globally (Duke and Powles, 2008). Originally, being a nonselective herbicide, its use was limited to vegetation management in noncrop areas; however, introduction of glyphosate-resistant (GR) crops in the late 1990s, coupled with their exceptional adoption, led to accelerated use totaling approximately 128 million ha worldwide in 2012 (James, 2012). GR crop technology has made a significant contribution to global agriculture and the environment, as it not only increased farm income by \$32.2 billion (Brookes and Barfoot, 2013), but also moderated the negative environmental impacts of mechanical weed management practices (Gardner and

Nelson, 2008; Bonny, 2011). Glyphosate offers a simple, effective, and economic weed management option in GR crops. In addition, it provides immense value in no-till crop production systems by enabling soil and moisture conservation. However, due to intensive glyphosate selection pressure, several weed populations globally have evolved resistance through a variety of mechanisms. Globally, herbicide resistance, in particular the recent proliferation of glyphosate resistance in weed species, is a major crop protection threat; nearly two dozen GR weed species have been reported in the last 15 years (Heap, 2014).

Glyphosate, an aminophosphonic analog of the natural amino acid Gly, nonselectively inhibits 5-Enolpyruvylshikimate-3-Phosphate synthase (EPSPS) in plants, preventing the biosynthesis of the aromatic amino acids Phe, Tyr, and Trp (Steinrücken and Amrhein, 1980), resulting in the death of glyphosate-sensitive individuals. In plants, EPSPS is one of the key enzymes in the shikimate pathway (Herrmann and Weaver, 1999), and glyphosate inhibits EPSPS by binding to EPSPS-shikimate-3-P binary complex forming an EPSPS-shikimate-3-P-glyphosate complex (Alibhai and Stallings, 2001). Bradshaw et al. (1997) hypothesized against the likelihood of weeds evolving resistance to glyphosate, primarily because of its complex biochemical interactions in the shikimate pathway and also due to the absence of known glyphosate metabolism

Figure 1. FISH mapping of *EPSPS* gene on chromosome of GS (A and C) and GR (B and D) *K. scoparia*. A, Three NOR sites on somatic metaphase chromosome pairs of GS1, one with a minor NOR signal showing faint *EPSPS* signal on the distal end. B, Somatic metaphase spreads of GR1 showing bright *EPSPS* signal on the same chromosome pair with a minor NOR site. FISH mapping of the *EPSPS* gene on somatic metaphase chromosomes of GS2 (C) and GR2 (D) *K. scoparia*. Arrows point to chromosomes with an *EPSPS* signal. Bar = 10 μm.

in plants. Nonetheless, several cases of glyphosate resistance, as a result of difference in glyphosate translocation (Preston and Wakelin, 2008) or mutations in the *EPSPS*, were confirmed (Baerson et al., 2002). More importantly, duplication/amplification of the *EPSPS* appears to be the basis for glyphosate resistance in several weeds (Sammons and Gaines, 2014). Here, we use duplication to refer to the formation of first repetition of a chromosomal segment and amplification to refer to increase in number of the repetitions (more than two repetitions of a chromosomal segment) under positive selection. The first case of *EPSPS* amplification as a basis for glyphosate resistance was reported in an *Amaranthus palmeri* population from GA (Gaines et al., 2010). In this *A. palmeri* population, there is a massive increase (>100-fold relative to glyphosate-susceptible [GS] plants) in *EPSPS* copies, and these copies are dispersed throughout the genome (Gaines et al., 2010).

Field-evolved GR *Kochia scoparia* populations were first reported in western Kansas in 2007 (Heap, 2014). We previously determined that evolution of GR populations of *K. scoparia* in the U.S. Great Plains is also due to amplification of the *EPSPS* (A. Wiersma and P. Westra, unpublished data). Unlike in GR *A. palmeri*, we found relative *EPSPS*:acetolactate synthase (*ALS*) copies ranging from three to nine in GR *K. scoparia* populations. While it quickly became widespread in the region, its presence was reported in another five Great Plains states by 2013 (Heap, 2014). GR *K. scoparia* populations we tested were 3- to 11-times resistant (population level) to glyphosate compared with a GS population (Godar, 2014), and *EPSPS* expression positively correlated with genomic *EPSPS* copy number (A. Wiersma and P. Westra, unpublished data). Here, we reveal the genomic organization of the amplified *EPSPS* copies in two GR *K. scoparia* populations, an

alternative mechanism of gene amplification to that reported in GR *A. palmeri*.

RESULTS

Chromosomal Location of the Amplified *EPSPS* Copies

To investigate the location of amplified *EPSPS* gene copies on GR *K. scoparia* chromosomes, we used fluorescence in situ hybridization (FISH). Analysis of FISH showed a marked increase in *EPSPS* signal in GR *K. scoparia* plants relative to GS plants (Fig. 1). In GS1 and GR1 *K. scoparia*, three chromosome pairs with nucleolus organizer regions (NORs) were detected, one of which, with a minor NOR signal, had the *EPSPS* gene on the distal end (Fig. 1, A and B). On metaphase spreads, the *EPSPS* probe detected much brighter signal on the GR1 chromosome pair relative to the signal on the GS1 chromosome pair (Fig. 1, A and B). Similar *EPSPS* signals were observed on metaphase chromosomes of GS2 and GR2 plants (Fig. 1, C and D). On prometaphase chromosomes and interphase nuclei, only a faint *EPSPS* signal was seen on each chromatid (Supplemental Fig. S1, A and C) on GS1 samples, whereas five to seven partially overlapping signals of the *EPSPS* probe could be distinguished at this location on GR1 samples (Supplemental Fig. S1, C and D).

High-Resolution Mapping of the *EPSPS* Cluster

To further explore the arrangement of *EPSPS* gene copies, we first performed FISH on stretched DNA fiber (Fiber FISH) on GS2 and GR2 plants using one-color
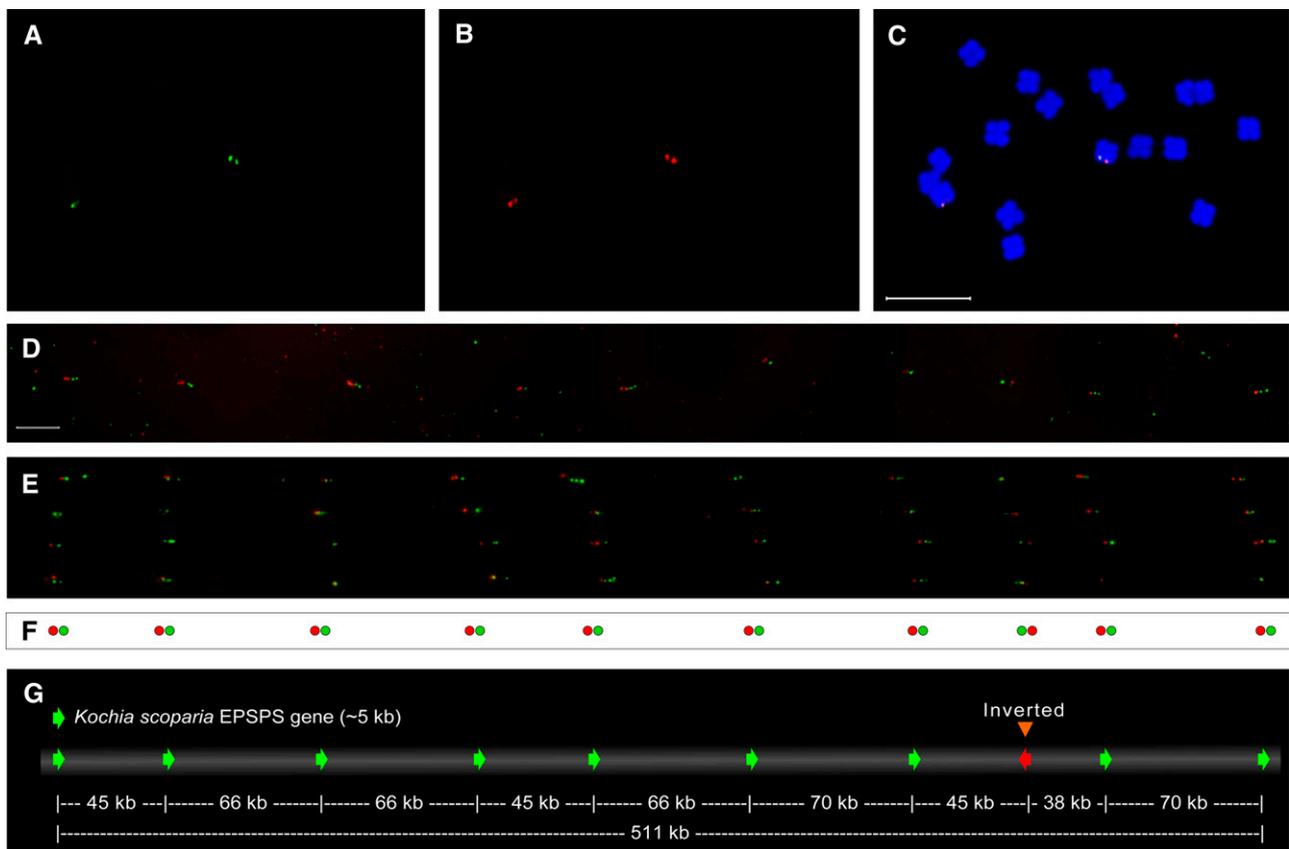
Figure 2. High-resolution Fiber FISH mapping of *EPSPS* of GR (GR2) *K. scoparia*. A to C, Metaphase chromosome spread in two-color FISH showing *EPSPS* copies clustered at the distal end of homologous chromosomes. D and E, Hybridization of two colored (red and green) *EPSPS* probes on five different chromosome fibers. F and G represent orientation of *EPSPS* copies and estimated distance between two adjacent *EPSPS* copies based on D and E. Red signal (approximately 1.9 kb) and green signal (approximately 2.5 kb) encompass the entire length of *EPSPS* in *K. scoparia*. Measurement of the cluster of *EPSPS* genes was 511.8 ± 26.0 kb (*n* = 7) in length. Bar = 10 μm.

probes followed by two-color probes. High-resolution images of Fiber FISH results showed only one *EPSPS* copy in GS2 plants (Supplemental Fig. S2A). In agreement with the brighter probe hybridization signal in FISH results (Fig. 1D), we observed tandemly configured 10 copies of *EPSPS* gene on a single DNA fiber of GR2 plants (Supplemental Fig. S2B). On metaphase chromosomes of the GR2 plant, the *EPSPS* probe hybridized to the same location, giving comparable signal intensity (Fig. 2, A–C) as previously observed in Figure 1C. The two-color probes on a single DNA fiber detected 10 *EPSPS* copies, one with an inverted *EPSPS* sequence (Fig. 2, D–F). The total length of the amplified region (measured on seven individual DNA fibers) is approximately 511 ± 26 kb. The *EPSPS* copies are located approximately 40 to 70 kb apart on a GR2 *K. scoparia* chromosome (Fig. 2G).

Inheritance of Glyphosate Resistance

We investigated the inheritance of glyphosate resistance in *K. scoparia* using a classical genetic approach.

We selected nonsegregating GS1 and GR1 lines (see "Materials and Methods") as parents to generate F1 progeny. F1 seed was successfully generated from reciprocal crosses. F2 seed was produced by self-pollinating F1 plants. In response to a field use rate of glyphosate (868 g acid equivalent [ae] ha^{-1}) application, the GS plants showed stunted growth and eventually died, while GR plants exhibited little or no injury and continued to grow normally (Fig. 3A). As expected for a nuclear-inherited *EPSPS* gene, F1 plants derived from either GR × GS or GS × GR crosses survived 868 g ae ha^{-1} glyphosate application (Fig. 3A). A total of 115 F2 plants were evaluated for glyphosate resistance with the same dose (868 g ae ha^{-1}) of glyphosate. F2 progeny segregated as 85 GR to 30 GS (three GR to one GS with χ^2 = 0.072), fitting a single-locus inheritance.

Segregation of *EPSPS* Copies in F1 and F2 Progeny

The *EPSPS* copies in parental plants (*n* = 2, GS1; *n* = 4, GR1), F1 (*n* = 6), and F2 progeny (*n* = 50) were determined using quantitative PCR (qPCR) on genomic
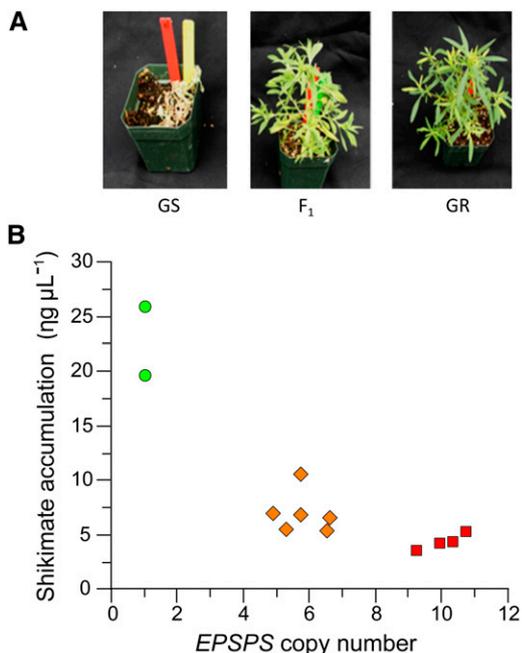
Figure 3. A, Response of GS, F1, and GR plants to a glyphosate rate of 868 g ae ha^{-1}. B, Relationship between *EPSPS* genomic copy number and shikimate accumulation in two GS (circles), four GR (squares), and six F1 plants (diamonds) of *K. scoparia*. *EPSPS* copy number and accumulation of shikimate were determined as described in "Materials and Methods."

DNA. GR1 parental plants possessed nine to 11 copies relative to GS1 plants (Fig. 3B). F1 progeny had approximately five to seven *EPSPS* copies (Fig. 3B). In F2 progeny, the copy number ranged from one to 13 at a frequency of 24% and 76% with one and four to 13 *EPSPS* copies, respectively. The *EPSPS* copy number observed in our genetic populations is in agreement with the inheritance of glyphosate resistance.

We also measured the amount of shikimate accumulated in glyphosate-treated leaf discs to estimate the level of glyphosate resistance. Shikimate accumulation results in plants when *EPSPS* is inhibited by glyphosate (Amrhein et al., 1980; Herrmann and Weaver, 1999), thus the degree of shikimate accumulation may be used as an indirect measure of a plant's sensitivity to glyphosate. In this case, if there are higher *EPSPS* copies, less shikimate accumulation was expected. GR parents accumulated less shikimate in leaf discs compared with GS parents (Fig. 3B). F1 plants accumulated drastically more shikimate than GS parents ($P < 0.001$) and less shikimate than GR parents ($P = 0.064$). Overall, shikimate accumulation correlated with the *EPSPS* copy number ($r = -0.841$).

K. scoparia Populations Show an Increase in *EPSPS* Copies and Level of Resistance over Years

We have been collecting field populations of *K. scoparia* over several years to test whether, in response to glyphosate application (selection event), there is an increase in *EPSPS* copy number due to recombination and selection at the *EPSPS* locus. We estimated the *EPSPS* copies and the level of glyphosate resistance in GR *K. scoparia* plants from populations that were collected in Kansas in 2007, 2010, and 2012. The results suggest that the GR *K. scoparia* plants collected in 2007 possessed an average of nine *EPSPS* copies, while plants from the 2010 and 2012 collection had up to 12 and 16 copies, respectively (Fig. 4). Furthermore, GR *K. scoparia* plants with nine and 12 copies withstood 1,736 g ae ha^{-1} but did not survive 3,472 g ae ha^{-1} glyphosate. However, plants from the 2012 collection survived glyphosate rates up to 5,208 g ae ha^{-1} (Fig. 4), implying a progression in *EPSPS* copies and the level of glyphosate resistance from 2007 to 2012. These results indicate that *K. scoparia* populations with increased *EPSPS* copies likely arose due to unequal crossing over and may have been favored by glyphosate selection.
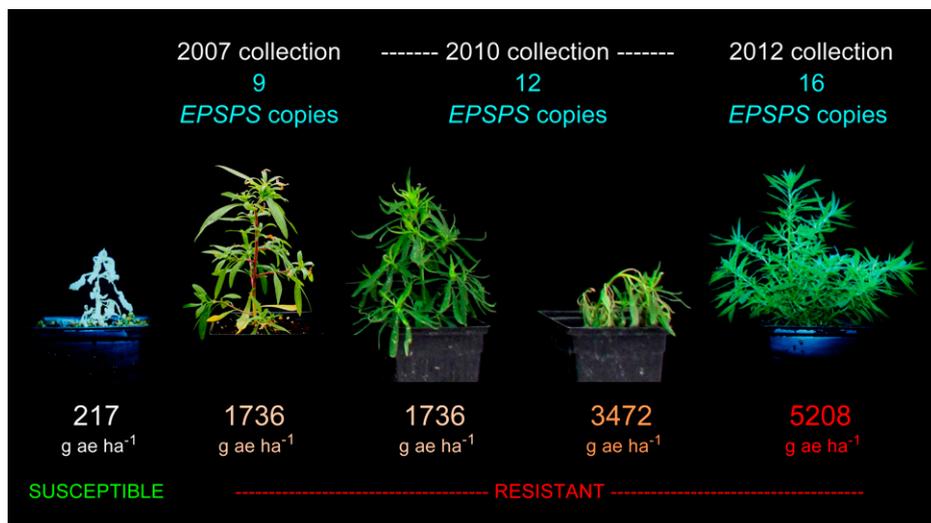
Figure 4. *EPSPS* genomic copies and level of glyphosate resistance in *K. scoparia* plants collected in 2007, 2010, and 2012.

DISCUSSION

This study reports the first case of tandem amplification of a target site as a mechanism of naturally evolving resistance to herbicides in plants. Massive amplification of the *EPSPS* gene randomly dispersed throughout the genome (Gaines et al., 2010), likely mediated by transposable elements (Gaines et al., 2013), has been recently reported in GR *A. palmeri*. Tandem amplifications of genes that metabolize insecticides have been reported in organophosphate-resistant populations of *Culex quinquefasciatus* mosquitoes (Paton et al., 2000) and *Myzus persicae* (Field and Devonshire, 1997). Our results demonstrate the tandem amplification of a target gene itself as a basis for mechanism of herbicide resistance. An intriguing question concerning the resistance mechanism to glyphosate is whether the *EPSPS* copy number increased in response to a positive selection or whether rare plants with multiple copies existed prior to selection. To date, at least one example of preexistence of the multicopy target gene has been reported, which results in resistance to kinase inhibitors used in lung cancer therapy (Turke et al., 2010).

In nature, gene duplication is a common phenomenon and is a precursor for genetic diversity (Wagner et al., 2007). The significance of gene duplication has been comprehensively reviewed (Van de Peer, 2004; Bailey and Eichler, 2006; Conant and Wolfe, 2008; Ponting, 2008). Some duplicated genes confer an immediate adaptive advantage (Perry et al., 2007) and provide a substrate for further amplification under selection, i.e. adaptive amplification. One of the common mechanisms of such amplification is unequal crossing over that takes place between homologs or sister chromatids within the amplified region (van Binsbergen, 2011). The role of unequal meiotic recombination in the formation of many disease resistance gene clusters in crop plants has been documented (Van der Hoorn et al., 2001; Nagy and Bennetzen, 2008; Luo et al., 2011). Continuous variation in *EPSPS* copy number and a positive correlation between *EPSPS* expression and the copy number that we have seen (A. Wiersma and P. Westra, unpublished data) suggest that the *EPSPS* copy number in *K. scoparia* plants increases through an adaptive process. Furthermore, hybridization of *EPSPS* probes at distal ends of homologous chromosomes of *K. scoparia* (Fig. 1) also suggests that an increase in *EPSPS* copies in GR *K. scoparia* may have occurred as a result of unequal crossover, as the gene duplication via unequal crossover most likely occurs at the telomere region of chromosomes (Royle et al., 1988; Amarger et al., 1998; Ames et al., 2008).

Here, we illustrate a model for *EPSPS* amplification via unequal crossover in response to glyphosate selection in *K. scoparia* (Fig. 5). Survival of the plant that inherits duplicated *EPSPS* copy (heterozygous for the duplicated copy) from the first duplication event is critical for evolution of resistance to glyphosate, as such an individual will have only a slightly elevated advantage under glyphosate selection. Factors including lower-than-normal use rates of glyphosate, environmental stress

(drought and high temperatures), and incomplete spray coverage may result in its survival, thereby allowing it to establish and reproduce one-fourth of the progeny homozygous for the duplicated copies. Sequence homology between the duplicated segments provides a substrate for unequal crossover to happen, leading to *EPSPS* copy number gain and loss in next-generation progeny. Under continuous selection, plants with higher *EPSPS* copies will be selected until the return to additional gain in the copy number reaches a plateau. Rate of copy number increase will depend on the interplay between the number of repetitions of sequence homology (copy number) and the return to the gain in additional copy number.

Models for predicting the spread of herbicide resistance in weeds largely rely on underlying genetic mechanisms (single versus polygenic, maternal versus nuclear inherited) and levels of resistance conferred in different states of zygosity. The single-locus inheritance of the *EPSPS* copies or glyphosate resistance we observed in our classical genetic population is in conformity
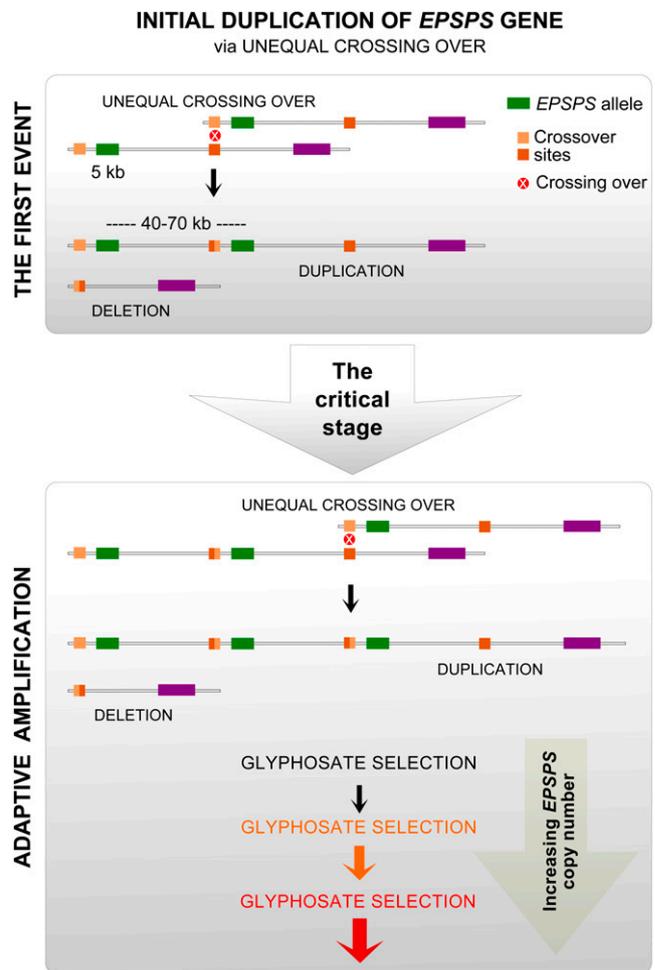
Figure 5. Illustration of a model suggesting *EPSPS* amplifications via unequal crossover in response to glyphosate selection and evolution of glyphosate resistance in *K. scoparia*.

with tandem arrangement of *EPSPS* copies. In the context of gene amplification, cytogenetic arrangement of the amplified gene (tandem versus dispersed) and their stability, as well as the magnitude of selection pressure, determine dynamics of the resistance locus (loci), not only at the population, but also at the individual level, unlike in classical genetic models. *EPSPS* transcript analysis showed that there was no polymorphism in *EPSPS* transcript sequences, and no *EPSPS* splice variants were detected in GR *K. scoparia* (A. Wiersma and P. Westra, unpublished data). Although, initially, most of the *EPSPS* copies, if not all, will have a complete set of functional motifs and be functionally indistinguishable from the original copy, over time, amplified sequences may diverge and code for new functions. Whether increase in *EPSPS* gene copies affects fitness of the plants in the absence of selection is unknown; however, a manyfold increase of *EPSPS* genes in GR *A. palmeri* does not accrue a measurable fitness cost (Giacomini et al., 2014; Vila-Aiub et al., 2014). These two factors, along with the magnitude of selection pressure, will shape the way for additional increases in the *EPSPS* copy number.

Our results revealed the tandem genomic organization of amplified *EPSPS* copies in two GR *K. scoparia* populations. Nonetheless, the significance of the inverted *EPSPS* copy in the evolution of glyphosate resistance remains unknown. Although our previous study shows similar levels of *EPSPS* amplification in other GR *K. scoparia* populations from the U.S. Great Plains, whether similar amplification patterns exist in those populations remains to be seen. Further investigation of flanking sequences, including breakpoints, will shed additional light on the mechanism of such possibly recurrent rearrangement events that resulted in the evolution of glyphosate resistance in *K. scoparia* populations.

MATERIALS AND METHODS

Plant Materials

Plants from two GS *Kochia scoparia* populations, GS1 and GS2, and two GR *K. scoparia* populations, GR1 and GR2, all from Kansas, were used in this study.

FISH Procedure

Somatic chromosome preparations (using the drop technique), direct probe labeling (by nick translation), and the FISH procedure on GS1 and GR1 plants were performed as described previously (Kato et al., 2004, 2006) with minor modifications. Root tips were collected from young plants and treated in a nitrous oxide gas chamber for 1.5 h, fixed on ice in cold 90% (v/v) acetic acid for 10 min, and washed and stored in 70% (v/v) ethanol at –20°C. For slide preparation, roots were washed in tap water for 10 min and then in KCl buffer for 5 min (75 mM KCl, 7.5 mM EDTA, pH 4). Seven meristems (0.5–1 mm long) were placed in 20 μL of 4% (w/v) cellulase Onozuka R-10 (Tokyo, catalog no. 201069) and 1% (w/v) pectolyase Y23 (Karlan, catalog no. 8006) in KCl buffer and incubated for 43 min at 37°C. Digested meristems were washed for 5 min in ice-cold Tris-EDTA buffer (pH 7.6), with three additional washes in 100% ethanol. Meristems were dispersed with a needle in 20 μL of an ice-cold acetic acid:methanol mix (9:1) and immediately dropped onto three precleaned glass slides placed in a humid chamber. Dried preparations were UV cross linked, soaked in methacarn solution (methanol:chloroform:glacial acetic acid [6:3:1]) for 1 min, dried, and used for hybridization on the same day. For labeling the NOR ribosomal RNA loci, clone pTa71, containing a 9-kb insertion with 18S, 5.8S, and 26S ribosomal RNA wheat (*Triticum aestivum*) genes and intergenic

spacers (Gerlach and Bedbrook, 1979), was used as a probe. Five microliters of probe mixture contained 200 ng of each *EPSPS* gene PCR product labeled with Texas Red-5-dCTP and 160 ng of pTa71 labeled with Fluorescein-12-deoxy-uridine triphosphate (PerkinElmer, catalog nos. NEL413001EA and NEL426001EA). The mixture of probes and the slide preparation were denatured at 100°C separately. The rest of the FISH procedure and washes were performed by using the method described by Kato et al. (2006).

The FISH on the somatic metaphase chromosome of GS2 and GR2 was performed using a procedure as described by Koo et al. (2010). Biotin- and digoxigenin-labeled probes were detected with Alexa Fluor 488 streptavidin antibody (Invitrogen) and rhodamine-conjugated anti-digoxigenin antibody (Roche Diagnostics), respectively.

In both FISH experiments, chromosome preparations were mounted and counterstained with 4',6-diamidino-2-phenylindole solution in Vectashield (Vector Laboratories, catalog nos. H–1200 and H–1300). FISH images were captured with a Zeiss Axioplan 2 microscope using a cooled CCD camera CoolSNAP HQ2 (Photometrics) and AxioVision 4.8 software (Zeiss). The final contrast of the images was processed using Adobe Photoshop CS5 software.

EPSPS FISH Probe Preparation

Sequences of *K. scoparia* *EPSPS* mRNA and the *Amaranthus palmeri EPSPS* gene (Gaines et al., 2010; GenBank accession no. JX56456) were used to develop the PCR primers (Supplemental Fig. S3; Supplemental Table S1). The *EPSPS* gene was amplified using PSI *K. scoparia* genomic DNA as a template isolated with Qiagen DNeasy Plant Mini kit (catalog no. 69104). The PCR reaction included JumpStart REDTaq ReadyMix (Sigma, catalog no. P0982), 0.4 μM of each primer, and 0.5 to 4 ng μL^{-1} template DNA. PCR cycles consisted of initial denaturation at 96°C for 5 min, 35 cycles at 96°C for 30 s, 57°C for 30 s, and 72°C for 4 min, and a final extension of 15 min. PCR products were cut and eluted from agarose gel with a Qiagen Gel Extraction kit (catalog no. 28706) and reamplified using the same primers. PCR products were purified with Invitrogen PCR Purification kit (catalog no. K3100–01) and verified by sequencing (Genewiz). The sequence of the amplified part of *K. scoparia EPSPS* gene was submitted to the National Center for Biotechnology Information GenBank database with accession number KJ374721. Three PCR products were tested separately by FISH, and products 1 and 3 that showed no background staining on *K. scoparia* chromosomes were used as a pooled FISH probe.

Fiber FISH Procedure

Young leaf tissues were collected from fast growing plants of GS2 and GR2. Nuclei isolation, DNA fiber preparation, and Fiber FISH were performed following published protocols (Jackson et al., 1998; Koo et al., 2011). Fiber FISH images were captured and processed as previously described in the FISH procedure. The cytological measurements of the Fiber FISH signals were converted into kilobases using a 3 kb μm^{-1} conversion rate.

EPSPS Fiber FISH Probe Preparation

Sequences of *A. palmeri EPSPS* gene (GenBank accession no. JX564536) were used to develop the PCR primers (Supplemental Fig. S4) for cloning *EPSPS* gene from *K. scoparia*. The probes were amplified with primers (Supplemental Table S1) using GS2 plant genomic DNA as a template. PCR product was cloned in 2.1-TOPO TA vector, and the clones were labeled with either biotin-16-UTP or digoxigenin-11-deoxyuridine triphosphate (Roche Diagnostics) using a standard nick translation reaction.

Creation of Genetic Populations and Their Phenotyping

Plants from *K. scoparia* populations GS1 and GR1 were grown in a greenhouse. Before flowering, the whole plant was covered with plastic bread bags (33 × 60 cm) with microperforations for self-pollination. Upon plant maturity, seed was harvested separately from individual plants. A subset of selfed progeny seeds were planted, and about 40 plants (8–10 cm tall) from each line were treated with glyphosate (Roundup WeatherMAX, Monsanto Company) at the rate of 868 g ae ha^{-1} using a chamber sprayer calibrated to deliver 187 L ha^{-1}. None of the GS1 plants survived the glyphosate treatment and were hence identified as a GS line. All GR1 plants survived the glyphosate treatment and were therefore considered for crossing as a GR line. Reciprocal crosses of GS1 and GR1 (R × S represents a GR female pollinated with GS pollen and vice versa for S × R)

plants were performed as follows. *K. scoparia* bears protogynous flowers with stigma being receptive for 1 week before anthesis of the same flower. Therefore, prior to stigma emergence, all the leaves and apical meristems were removed from a few randomly selected branches of GR or GS plants and covered with Lawson 217 pollination bags. After stigma emergence, using a sterile forceps, pollen from the dehisced anthers of GR or GS plants (chosen as male parents) was transferred separately onto the stigmas of the maternal flowers. Immediately after pollination, the flowers were covered with the same pollination bags for 10 d. Mature F1 seed was harvested separately from reciprocal crosses. F1 plants were treated with glyphosate as described above, and all F1 plants from both reciprocal crosses survived a glyphosate rate of 868 g ae ha^{-1}. Randomly selected F1 plants were transplanted into larger pots and self-pollinated (using the plastic bread bags with microperforations as mentioned earlier), and F2 seed was harvested separately. F2 plants were treated with a glyphosate rate of 868 g ae ha^{-1} as described above.

Shikimate Accumulation Assay

An in vivo measure of shikimate accumulation was determined on parental, F1, and F2 progeny following the procedure developed by Shaner et al. (2005). Six 4-mm leaf discs were collected from a fully expanded young leaf of a single plant when 8 to 10 cm tall. Discs were placed in a 96-well plate with one disc per well containing 100 μM glyphosate solution. Leaf discs were incubated for 16 h under continuous light (μmol 250 m^{-2} s^{-1}). A shikimate standard curve was used to calculate the shikimate accumulation in ng shikimate μL^{-1}. The experiment was done in triplicate and repeated.

qPCR

EPSPS gene copy number was determined in parental, F1, and F2 plants. Leaf tissue samples were collected in 1.5-mL microcentrifuge tubes and immediately frozen in liquid nitrogen and stored at –20°C. DNA was extracted using the Qiagen DNEasy Plant Mini Kit and quantified using a NanoDrop spectrophotometer. The relative *EPSPS* gene copy number was determined by qPCR on genomic DNA using *ALS* as a reference gene. *EPSPS* forward and reverse primers were 5'-GGCCAAAAGGGCAATCGTGGAG-3' and 5'-CATTGCCGTTCCCGCGTTTCC-3', respectively. ALS forward and reverse primers were 5'-ATGCAGACAATGTTGGATAC-3' and 5'-TCAACCATCGATACGAACAT-3', respectively. These primers produce products of 102 and 159 bp for *EPSPS* and *ALS*, respectively. qPCR was performed using 96-well plates, with each well containing a master mix comprised of 10 μL of iQ SYBR Green Super Mix (BioRad), 1 μL of each corresponding forward and reverse primer, 16 ng of genomic DNA, and 4 μL of deionized water. Each reaction was done in triplicate and was repeated. PCR cycle parameters were set for 95°C for 3 min, denaturing was set at 95°C for 10 s, annealing and extension were set at 60°C for 30 s, and the denaturing/annealing steps were repeated for 39 cycles. Relative quantification of *EPSPS* was calculated as ΔCt = Ct$_{ALS}$ – Ct$_{EPSPS}$, where Ct$_{ALS}$ and Ct$_{EPSPS}$ are the threshold cycles for *ALS* and *EPSPS* genes, respectively, and *EPSPS* copy number was expressed as $2^{\Delta Ct}$ (Gaines et al., 2010). The copy number was averaged across replications, and the SD was calculated for each plant sample.

EPSPS Copies and Level of Resistance in *K. scoparia* Populations Collected over Years

EPSPS copies and the level of resistance to glyphosate were determined in *K. scoparia* populations collected over years. Seed of GR *K. scoparia* was collected from fields in 2007, 2010, and 2012. Levels of resistance to glyphosate on 20 to 32 plants from each collection were determined by shikimate accumulation assay and/or treating whole plants with varying rates of glyphosate (0–5,208 g ae ha^{-1}) as previously described. *EPSPS* copy number of at least six plants from each collection that showed highest levels of resistance was determined using qPCR as previously described.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number KJ374721.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. FISH mapping of the *EPSPS* gene on chromosome of glyphosate-susceptible GS1 and glyphosate-resistant GR1 *K. scoparia*.

Supplemental Figure S2. High resolution Fiber FISH results showing chromosomal distribution *EPSPS* copies in GS and GR *K. scoparia* plants.

Supplemental Figure S3. Part of the *K. scoparia* *EPSPS* sequence used for FISH probe production and sequencing.

Supplemental Figure S4. *EPSPS* structure of *A. palmeri* and PCR primer positions for *K. scoparia* *EPSPS* cloning for FISH and Fiber FISH probe preparation.

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

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