

Gene Duplication and Aneuploidy Trigger Rapid Evolution of Herbicide Resistance in Common Waterhemp¹[OPEN]

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An increase in gene copy number is often associated with changes in the number and structure of chromosomes, as has been widely observed in yeast and eukaryotic tumors, yet little is known about stress-induced chromosomal changes in plants. Previously, we reported that the *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) gene, the molecular target of glyphosate, was amplified at the native locus and on an extra chromosome in glyphosate-resistant *Amaranthus tuberculatus*. Here, we report that the extra chromosome is a ring chromosome termed extra circular chromosome carrying amplified *EPSPS* (ECCAE). The ECCAE is heterochromatic, harbors four major *EPSPS* amplified foci, and is sexually transmitted to 35% of the progeny. Two highly glyphosate resistant (HGR) *A. tuberculatus* plants with a chromosome constitution of $2n = 32+1$ ECCAE displayed soma cell heterogeneity. Some cells had secondary ECCAEs, which displayed size polymorphisms and produced novel chromosomal variants with multiple gene amplification foci. We hypothesize that the ECCAE in the soma cells of HGR *A. tuberculatus* plants underwent breakage-fusion-bridge cycles to generate the observed soma cell heterogeneity, including de novo *EPSPS* gene integration into chromosomes. Resistant soma cells with stable *EPSPS* amplification events as de novo insertions into chromosomes may survive glyphosate selection pressure during the sporophytic phase and are plausibly transmitted to germ cells leading to durable glyphosate resistance in *A. tuberculatus*. This is the first report of early events in aneuploidy-triggered de novo chromosome integration by an as yet unknown mechanism, which may drive rapid adaptive evolution of herbicide resistance in common waterhemp.

Herbicides are widely used in the management of weeds in agricultural production systems. Among the many classes of herbicides, glyphosate [*N*-(phosphonomethyl) Gly], popularly known as Roundup, is widely used in agriculture, especially on glyphosate-

resistant (GR) transgenic crops. Vast adoption of GR crops across the globe and overreliance on glyphosate as a single means of weed control has spawned many GR weeds (Fernando et al., 2016). Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) enzyme of the shikimate pathway, resulting in depletion of aromatic amino acid synthesis and, eventually, plant death (Amrhein et al., 1980; Steinrücken and Amrhein, 1980; Duke and Powles, 2008). Overexpression of the *EPSPS* gene as a result of gene amplification can lead to the evolution of GR weeds (Gaines et al., 2010; Salas et al., 2012; Jugulam et al., 2014; Malone et al., 2016; Molin et al., 2017). The *EPSPS* gene amplification may be local (Jugulam et al., 2014) or is postulated to be dispersed across the genome (Gaines et al., 2010; Molin et al., 2017).

Recently, we reported local amplification in low to moderately resistant plants and, surprisingly, an extra chromosome harboring amplified *EPSPS* copies in highly glyphosate-resistant (HGR) plants of *Amaranthus tuberculatus* (Dillon et al., 2017). Plants with the extra chromosome were correlated with increased expression of the *EPSPS* gene (Dillon et al., 2017).

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Molecular marker analysis showed that the extra chromosome originated from the autosomes; however, little else is known about the extra chromosome because it was only analyzed in mitotic preparations when chromosomes are highly condensed. Here, we report the cytogenetic structure of this chromosome using highly extended prometaphase chromosomes as well as sexual transmission, and the nature and mechanisms by which this extra chromosome is acquired and may disperse amplified *EPSPS* gene copies across the genome.

RESULTS

HGR Plants Are Aneuploid, Involving a Ring Chromosome with Amplified *EPSPS* Gene Copies

An HGR plant with 14 *EPSPS* copies had $2n = 33$ chromosomes, and the extra chromosome was similar in size to the other chromosomes (Fig. 1A). Fluorescence in situ hybridization (FISH) mapping with the *EPSPS* gene probe showed one pair of chromosomes with signal in the centromeric region (Fig. 1A, arrowheads) and distinct signals on the extra chromosome (Fig. 1A, arrow). The 4',6-diamidino-2-phenylindole (DAPI)-stained relatively long prometaphase chromosomes revealed that the extra chromosome is a ring chromosome (Fig. 1B) and showed four distinct clusters of *EPSPS* foci (Fig. 1, C and E) similar to the signals in the condensed mitotic metaphase stage (Fig. 1A). The linear chromosomes are protected at their ends by telomeric DNA repeats (TTTAGGGn), as shown by strong FISH signals at the telomeric ends using the *Arabidopsis* telomeric DNA probe (Fig. 1F). The ring chromosomes have no free ends and do not need telomeres. As expected, telomeric DNA signals were not detected in the extra chromosome (Fig. 1D and arrow in Fig. 1F), confirming the circular structure of this extra

chromosome. We designated it as an extra circular chromosome carrying amplified *EPSPS* (ECCAE).

Next, we performed immunofluorescence using an antibody specific to histone H4 acetylation at Lys 5 (H4K5Ac), a hallmark of euchromatin. Strong immunofluorescence signals were detected on both ends of all metacentric chromosomes, or one end of all acrocentric chromosomes except the ECCAE (Fig. 1G). These results suggested that ECCAE is composed of heterochromatin and is derived from the pericentromeric region of a specific chromosome with amplified *EPSPS* copies. Because heterochromatin is relatively genetically inert, aneuploidy for such a heterochromatic chromosome has little effect on plant fitness (see next section). The ECCAE was observed in every metaphase cell. Thus, the ECCAE may have a functional centromere because it was transmitted stably during mitotic divisions.

ECCAE Is Sexually Transmitted to Progeny Plants

To study the sexual transmission of the ECCAE, we made crosses between an HGR plant with 14 *EPSPS* copies carrying the ECCAE (Supplemental Fig. S1A) and a glyphosate-susceptible (GS) plant with one *EPSPS* copy (Supplemental Fig. S1B). A total of 17 F1 plants were analyzed for *EPSPS* copy number variation and ECCAE transmission. The qPCR-based analysis revealed that 11 plants contained three to five copies of the *EPSPS* gene, four plants had seven to eight copies, and two plants had 12 copies (Fig. 2). Cytological analysis of these F1 plants showed that the 11 plants with three to five *EPSPS* copies did not have the ECCAE (Supplemental Fig. S1C) and the six F1 plants with 7 to 12 *EPSPS* copies had one copy of the ECCAE (Fig. 2; Supplemental Fig. S1, D and E). The transmission rate of ECCAE to the offspring was almost 35%, which is higher than the expected rate of 25% and

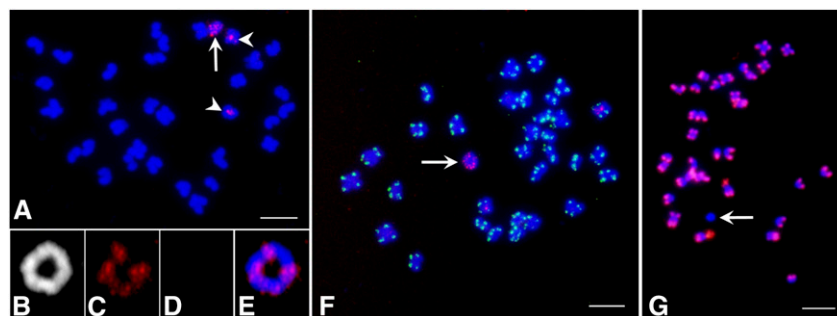
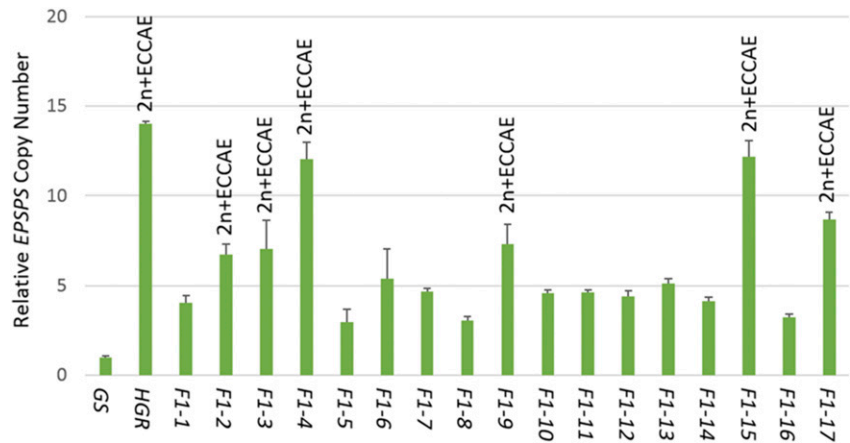


Figure 1. Cytogenetic characterization of an ECCAE. An HGR *A. tuberculatus* plant showed chromosome constitution of $2n = 32+1$ ECCAE (arrow); note *EPSPS*-FISH signals on the ECCAE and in the centromeric region of two other chromosomes (arrowheads in A). The ring structure of the ECCAE is clearly visible by DAPI staining at mitotic prometaphase stage (B). FISH signals of four *EPSPS* gene clusters in the ECCAE (C). The ECCAE lacks telomeric DNA FISH signal (D). Merged image of the ECCAE showing a circular structure and the *EPSPS* gene cluster (red; E). Two-color FISH using the telomeric DNA repeat probe did not detect any hybridization on the ECCAE (arrow), but strongly hybridized to the telomeres of all other chromosomes of the complement (green signals; F). Immunofluorescence of H4K5Ac on a HGR *A. tuberculatus* labeled the euchromatic regions of all the chromosomes except the ECCAE (arrow; G). Bars = 5 μ m.

Figure 2. qPCR analysis of *EPSPS* copy number in the parents and 17 F1 plants. Aneuploids detected by cytological analysis in the F1 progeny are also shown at the top of vertical bars. The vertical bars represent the relative β -*tubulin*:*EPSPS* gene copy number.



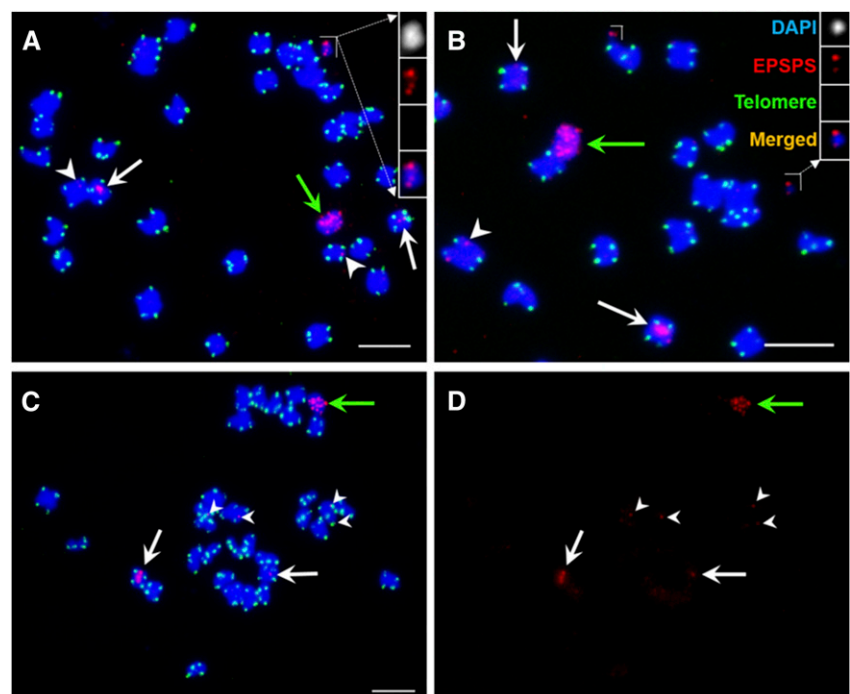
similar to that of other such types of extra chromosomes of recent origin (Dhar et al., 2002). The lack of an adverse effect of the ECCAE on gamete function and, hence, plant fitness, further reinforces the heterochromatic nature of the ECCAE.

The ECCAE Chromosome Is the Source of Soma Cell Heterogeneity for ECCAE Size, Number, and Random *EPSPS* Chromosome Integration Events in Meristematic Tissues of the Sporophyte

We assayed the chromosome constitution of six F1 plants with 2n+1 ECCAE using root meristematic tissues for ring chromosome structure and *EPSPS* gene amplification events. Cytological analysis of the four F1 plants with seven to eight *EPSPS* copies showed a chromosome

constitution of 2n = 32+1 ECCAE in all cells of the meristematic tissues (Fig. 1F; Supplemental Fig. S1D). However, two F1 plants with 12 *EPSPS* copies revealed highly variable sizes and numbers of ECCAEs and *EPSPS*-FISH patterns among different metaphase cells in a single root tip meristem preparation (Fig. 3; Supplemental Fig. S1E). The chromosome constitution of a sample of 50 cells from one root meristem was analyzed for plant F1-15 (Fig. 3). The results revealed that all the cells contained one pair of *EPSPS*-carrying chromosomes (heteromorphic *EPSPS* hybridization signals; white arrows in Fig. 3) and an intact ECCAE derived from the parents (green arrows in Fig. 3). In 28% of the cells, in addition to the standard ECCAE, we observed one or two secondary ECCAEs (sECCAEs) of intermediate (inset in Fig. 3A) or very small size (insets in Fig. 3B). Dual-color FISH with a telomeric DNA probe

Figure 3. Somatic cell chromosomes of plant F1-15 labeled with a telomeric DNA probe (green) and an *EPSPS* probe (red). Note the size variation in the sECCAEs and lack of telomeric signals (insets in A and B). In addition to the expected pericentromeric *EPSPS* signals on two chromosomes (white arrows), de novo formed *EPSPS*-FISH foci (arrowheads) were observed on an additional one to four chromosomes in 90% of the cells (A–D). Green arrows point to the ECCAE. Bars = 5 μ m.



gave negative results, indicating that the sECCAEs did not contain telomeres (insets in Fig. 3, A and B) and hence were ring chromosomes.

Most surprisingly, 90% of the cells revealed unambiguous *EPSPS*-FISH signals on a number of chromosomes; in addition, a wild-type signal was on one pair of chromosomes (arrowheads in Fig. 3). Most of these additional FISH signals were not located in the centromeric regions and thus were different from the wild-type *EPSPS* gene location in the centromeric region of one pair of chromosomes (arrowheads in Fig. 1, A and C). The number of these additional *EPSPS*-carrying chromosomes ranged from 1 to 4 in different cells (Fig. 3). These additional chromosomes, carrying *EPSPS* gene copies as well as the sECCAEs, were only detected in F1 plants that contained an ECCAE, suggesting that the ECCAE was the source of *EPSPS* genes for the observed chromosomal integration events.

DISCUSSION

Aneuploidy in Response to Glyphosate Selection Pressure

Aneuploidy, the gain of whole chromosomes, or segmental aneuploidy are the most prevalent events for genome adaptation to stressful conditions in yeast and mammalian cells, including cancer cells (Pavelka et al., 2010; Gordon et al., 2012; Yona et al., 2012). These extra chromosome copies usually contain genes needed to withstand a given stressful condition. Yona et al. (2012) reported that duplication of yeast chromosome III (trisomy) is acquired as a solution to heat stress. Two extra copies of chromosome 5L (iso5L) of *Candida albicans* were found to occur in response to antifungal drug selection (Selmecki et al., 2008). Cytogenetic abnormalities, including aneuploidy, have been found in cancer cells. In cancer cells, aneuploidy with the specific chromosomal duplication usually contains the chromosomal regions that harbor growth-promoting genes (Gordon et al., 2012). Thus, aneuploidy is likely a form of stress-inducible mutation in eukaryotes (Chen et al., 2012), but genome adaptation through aneuploidy may also occur spontaneously. To our knowledge, this and our earlier results (Dillon et al., 2017) are the first reports of aneuploidy-mediated gene amplification and adaptive response to herbicide glyphosate selection pressure in higher plants. But how are such aneuploids generated in the first place?

A primary aneuploid, trisomic for a chromosome carrying an amplified gene, may be acquired spontaneously through nondisjunction or chromosome mis-segregation as was shown in duplication of yeast chromosome III, which may have selective advantage under heat stress (Yona et al., 2012; see proposed model below). In cancer cells, defects in genes involving the spindle assembly checkpoint and chromatid cohesion are known to cause aneuploidy (Gordon et al., 2012). Chromosome loss after polyploidization also is reported to lead to high levels of aneuploidy in *Drosophila melanogaster* rectal cells (Schoenfelder et al., 2014).

Dhar et al. (2002) reported that the aneuploid triplo 2, trisomic for chromosome 2 of *Plantago lagopus*, resulted in rapid chromosomal changes in the extra copy of chromosome 2 leading to the formation of various types of ring or isochromosomes. Similar chromosomal structural changes were reported for extra copy of triplo 1L in barley (*Hordeum vulgare*; Singh, 2003). Alternatively, univalents at meiosis usually undergo anaphase lagging or centric misdivision giving rise to broken chromosomes at centromeric regions (Darlington, 1939). Aneuploid midget chromosomes were recovered from breakages in pericentromeric regions (Lukaszewski, 1997).

We propose the following steps for the origin of ECCAE. Chromosomal duplication (trisomy) involving chromosome-harboring amplified *EPSPS* may have occurred spontaneously (Fig. 4A). Aneuploidy tends to destabilize chromosomes (Darlington, 1939; Lukaszewski, 1997; Dhar et al., 2002; Singh, 2003; Passerini et al., 2016), and this may have triggered breakage of the chromosome

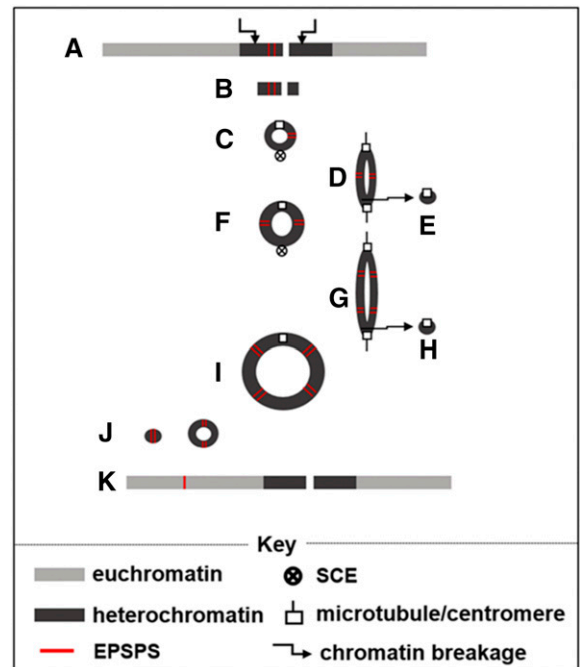


Figure 4. A proposed model for elucidating the BFB-mediated *EPSPS* gene amplification in GR *A. tuberculatus*. An extra chromosome carrying an amplified *EPSPS* may occur spontaneously and may be selected in response to glyphosate stress (A). The ring chromosome was formed by chromatin breakages in the proximal region of chromosome containing amplified *EPSPS* copies (B), followed by fusion of the proximal ends into a ring chromosome (C). Following chromosome replication, subsequent sister chromatid exchange (C) produce a double-sized dicentric ring chromosome (D). The dicentric ring chromosomes, following chromatid breakages at anaphase, produced different sized ring chromosomes, with (F) or without *EPSPS* genes (E). Additional rounds of BFB cycle (F–H) will produce ring chromosomes, such as the ECCAE observed (I). The ECCAE also produced sECCAEs with varying numbers of *EPSPS* gene copies (J; also shown in Fig. 3, A and B). The sECCAEs may undergo ectopic recombination with nonhomologous chromosomes and produce additional *EPSPS* foci in the genome (K).

around the centromere, including the amplified *EPSPS* locus (Fig. 4B). Broken chromosomes lacking telomeres are unstable but can be stabilized by the fusion of broken ends to form a ring chromosome (McClintock, 1938; Fig. 4C). We propose that ECCAE may have originated by this mechanism because no telomeric sequences were observed. Alternatively, the segmental duplications in the pericentromeric region may undergo ectopic recombination producing a ring chromosome (Appels et al., 1998), another plausible mechanism for the origin of a ring chromosome. We propose that such primordial ECCAEs with amplified *EPSPS* foci (Fig. 4C) had a selective advantage under glyphosate stress. Further selection pressure drove the evolution ECCAE; it acquired additional *EPSPS* foci and also grew in size as small chromosomes tend to be unstable and are lost during mitotic divisions (see next section).

A Model for the Evolution of ECCAE by Chromosome Breakage-Fusion-Bridge Cycles

Ring chromosomes can undergo breakage-fusion-bridge (BFB) cycles and produce different sized ring chromosomes and other chromosomal changes in somatic tissues during the sporophytic phase (McClintock, 1938; Gisselsson, 2001). We failed to detect BFB events due to lack of reliable anaphase cell spreads in our root tip preparation. Nonetheless, the ECCAE observed in this study very likely evolved through BFB cycles involving the primordial ECCAE (Fig. 4, C–I). Sister chromatid exchanges (Fig. 4C) in ring chromosomes frequently produce dicentric chromosomes (Fig. 4D), doubling the size of the original ring chromosome. The dicentric ring chromosomes suffer chromatid breaks at anaphase (Fig. 4D) and produce different sized ring chromosomes (Fig. 4, E and F). Additional rounds of the BFB cycle (Fig. 4, F–H) will produce ring chromosomes such as the ECCAE as observed in this study (Fig. 4I). Thus, ECCAE behavior provides an intrinsic mechanism of gene amplification and somatic cell heterogeneity for ECCAE polymorphisms. The glyphosate selection pressure led to the selection of observed ECCAE in HGR *A. tuberculatus*. However, as discussed above, ECCAEs are intrinsically unstable and also are transmitted only to a portion of the progeny. The GR *A. tuberculatus* plants in the absence of selection pressure will revert to sensitivity. How can ECCAE trigger more durable evolution of herbicide resistance?

ECCAE as a Trigger for de Novo Chromosome Integration Events and Potentially More Stable Herbicide Resistance

The dynamics of variation in *EPSPS* copy number and spread to additional chromosomes were revealed by pedigree analysis of two F1 plants with 12 *EPSPS* copies. Along with one large and one small *EPSPS* hybridization site in the pericentromeric region in one chromosome pair (arrows in Fig. 3), one to four

unambiguous de novo *EPSPS*-FISH foci were detected in most of the cells (arrowheads in Fig. 3). This suggested that ECCAE during mitotic divisions in soma cells was the source of de novo *EPSPS* gene amplification. The size of the signal in the de novo chromosome insertions was much smaller than the size of the *EPSPS* loci in the ECCAE. Thus, we can rule out integration of wild-type ECCAE in different chromosome integration events. Our results suggest at least two mechanisms of de novo chromosome insertion of *EPSPS* foci. First, tiny sECCAEs with a few *EPSPS* loci may insert into chromosomes by ectopic recombination. Second, ECCAE and sECCAE may be the source of extra circular DNAs or episomes. For example, Molin et al. (2017) have documented an *EPSPS* cassette that may be extra circular DNAs. The episomes may undergo ectopic recombination with nonhomologous chromosomes and reintegrate into other chromosomes as postulated in the deletion-plus-episome model (Maurer et al., 1987; Carroll et al., 1988; Wahl, 1989) and produce additional foci in the genome. Taken together, our results demonstrate that the formation of ECCAE may trigger an endless chain of BFB events, thereby constantly generating the novel chromosomal integration events (see Fig. 4) that may eventually lead to durable glyphosate resistance in *A. tuberculatus*.

CONCLUSION

McClintock (1984) predicted the existence of innate systems in sporophytic genomes that may rapidly respond to challenge. We have shown that chromosome mechanisms, such as ECCAE, exist in the sporophytic genomes that can rapidly generate copy number variation for the target gene and soma cell heterogeneity. This soma cell heterogeneity, upon selection pressure in the presence of herbicide, leads to selection of acquired herbicide resistance during the sporophytic phase and is transmitted to germ cells. The evolution of stable herbicide resistance is a multistep process. The first step is a local tandem gene amplification event of the *EPSPS* gene, the molecular target of herbicide action (Dillon et al., 2017). We postulate that the *EPSPS* amplification event and the intrinsic property of the chromosome segment where it is located triggered an aneuploidy event. The aneuploidy in response to glyphosate selection pressure on the sporophyte triggered somatic cell heterogeneity, including de novo *EPSPS* gene integration events. We hypothesize that de novo chromosome integration events are the early steps toward the evolution of more durable herbicide resistance.

MATERIALS AND METHODS

Production of F1 Progeny by Crossing GS and GR *Amaranthus tuberculatus* and Estimation of *EPSPS* Copies in F1 Progeny

A male plant of GS ($2n = 32$, with no ECCAE) and female HGR ($2n = 33$, with one copy of ECCAE) of *A. tuberculatus* were grown individually in Miracle-Gro potting mix in 10-cm³ plastic pots and watered from the top in a greenhouse (25/20°C temperature; 15/9-h light day/night, supplemented with 120 mmol m⁻² s⁻¹ illumination using sodium vapor lamps). After flower initiation, the

inflorescences of GS (male) and HGR (female) plants were covered together with a plastic bread bag (33 × 60 cm) containing microperforations. Upon plant maturity, seed were harvested from the HGR *A. tuberculatus* plant. Seed of this cross were planted and about 25 progeny were generated. The root tips from 17 F1 plants were harvested for FISH analysis. Additionally, leaf tissues from these 17 plants were harvested for DNA extraction and *EPSPS* copy determination.

The qPCR reaction was performed using a StepOnePlus real-time detection system (Applied Biosystems) to determine the *EPSPS* gene copy number in the 17 F1 *A. tuberculatus* plants. The qPCR reaction mix consisted of 8 μL of SYBR Green mastermix (Thermo Fisher Scientific), 2 μL each of forward and reverse primers (5 μM), and 2 μL of genomic DNA (20 ng/μL) to make the total reaction volume up to 14 μL. *EPSPS* gene copy number was measured relative to the *β-tubulin* gene (reference gene). PCR conditions were 95°C for 15 min and 40 cycles of 95°C for 30 s and 60°C for 1 min. A melt curve profile was included following the thermal cycling protocol to determine the specificity of the qPCR reaction. The following primer sequences were used: *EPSPS* F 5'-ATGTTG-GACGCTCTCAGAACTCTTGGT-3' and *EPSPS* R 5'-TGAATTTCTCCAG-CAACGGCAA-3'; *β-tubulin* F 5'-ATGTGGATGCCAAGAACATGATGTG-3'; and *β-tubulin* 5'-TCCACTCCACAAAGTAGGAAGAGTTCT-3'. The *EPSPS* gene copy number was measured with three technical replicates. Gene copy number was determined using the 2^{-CT} method, where CT is the threshold cycle and ΔCT is CT_{target gene} (*EPSPS*) – CT_{reference gene} (*β-tubulin*).

FISH and Immunofluorescence

Molecular cytogenetic mapping of *EPSPS* gene and telomeric DNA followed published protocols (Koo and Jiang, 2009; Dillon et al., 2017). To prepare the *EPSPS* FISH probe, the sequence of *A. tuberculatus EPSPS* mRNA (GenBank accession no. FJ869881) was used to develop the PCR primers (forward, 5'-GCCAAGAAACAAAGCGAAAT-3'; reverse, 5'-TTTCAGCATCATAATT-CATAACCC-3'; product length 1,804 bp). The PCR product was cloned in 2.1-TOPO TA vector (Invitrogen), and the clone was labeled with digoxigenin-11-deoxyuridine triphosphate (Roche Diagnostics) using a standard nick translation reaction. The clone, *Arabidopsis (Arabidopsis thaliana)* telomeric DNA (TTTAGGGn; Koo et al., 2016) was labeled with biotin-16-dUTP (Roche). Digoxigenin- and biotin-labeled probes were detected with rhodamine-conjugated antidigoxigenin (Roche) and Alexa Fluor 488 streptavidin (Invitrogen), respectively. The conditions for hybridization of antibody specific to H4K5Ac, detection of fluorescent signal, and image analysis were carried out as described (Jin et al., 2004).

Image Analysis

Chromosomes were counterstained with DAPI in Vectashield antifade solution (Vector Laboratories). The images were captured with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy) using a cooled CCD camera CoolSNAP HQ2 (Photometrics) and AxioVision 4.8 software. The final contrast of the images was processed using Adobe Photoshop CS5 software.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number FJ869881.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Inheritance of ECCAE in crosses of a highly glyphosate resistant *A. tuberculatus*, with glyphosate resistant *A. tuberculatus*.

Supplemental Figure S2. Two-color FISH detection of the *EPSPS* gene and telomere-specific probes.

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