Supporting Information

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Fig. S1. Two-color FISH mapping of the *EPSPS* gene (red signals) and eccDNA (green signals) on mitotic metaphase chromosomes of GS *A. palmeri* with one *EPSPS* copy (*A–D*) and *A. palmeri* with 12 *EPSPS* copies (*E–H*). (C and *D*) Pericentromeric location of the *EPSPS* gene in GS *A. palmeri* with one *EPSPS* copy. (G and *H*) Pericentromeric location of amplified *EPSPS* genes in *A. palmeri* with 12 *EPSPS* copies. In GS *A. palmeri*, eccDNA did not generate distinct hybridization signals (*B*), but significantly more intense signals (arrowheads) were detected at the amplified *EPSPS* gene locus in *A. palmeri* with 12 *EPSPS* copies (*F*). Arrowheads point to hybridization signals. (Scale bar, 10 µm.)



Fig. S2. (A) Validation of the ~400-kb BAC contig assembly using fiber-FISH. (B) BACs in pairs were used in fiber-FISH to verify their orientation. The six BACs were pooled, and the probe was then used in fiber-FISH to visualize the wild-type circular structure of eccDNA (C) and the polymorphic linear form (D).





Fig. S3. (*A*) Determination of *EPSPS* copy number of F_1 progeny derived from male GS x female GR *A. palmeri*. Palmer amaranth glyphosate susceptible [PA SUSC (GS)] and Palmer amaranth glyphosate resistant [PA RES (GR)] were used as controls. The *y* axis represents the relative β -tubulin/*EPSPS* gene copy number. (*B*) FISH mapping of eccDNA (red signals) on mitotic metaphase chromosomes and interphase nuclei in plant FHMS 6. Similar FISH patterns were detected on the metaphase cell spreads in all F_1 plants. Arrow indicates the interphase cell with no eccDNA hybridization signal. Arrowheads point to metaphase cells. (Scale bar, 10 μ m.)



Fig. S4. FISH mapping of eccDNA (red signals) on tapetum (A) and pachytene (B) cells of plant MHFS 1. (Scale bars, 10 μm.)

Table S1.	EPSPS gene copy number in GS and GR A. palmeri
plants	

Samples	EPSPS genomic copy number (β -tubulin as endogenous control)
GS	1*, 12*, 14, 18, 20
GR	62*, 78, 80*, 90, 120

Relative *EPSPS/β-tubulin* gene copy number was adjusted to 1 for known GS *A. palmeri*, and the copy numbers for GR *A. palmeri* plants shown here are relative to the GS *A. palmeri*.

*Male plant that was used for both mitotic and meiotic chromosome analysis. Some of female plants were also used in FISH.