Supporting Information

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SI Materials and Methods

Protein Extraction. Young expanding leaf tissue was sampled from selected R, S, and F_2 plants and frozen immediately in liquid nitrogen. The tissue was ground to a fine powder with 1% polyvinylpyrrolidone and transferred into a beaker containing extraction buffer [100 mM 3-(N-morpholino) propanesulfonic acid (Mops), 5 mM EDTA, 10% glycerin, 50 mM KCl, 0.5 mM benzamidine, 7.3 μM pepstatin, 25 mg/L trypsin inhibitor, and 4.2 μM leupeptin] and 5 mM β-mercaptoethanol. All subsequent extraction steps were carried out at 4°C. The mixture was homogenized with stirring, then centrifuged at 7,500 × *g* for 40 min. The supernatant was decanted through cheesecloth, then $(NH_4)_2SO_4$ was slowly added to reach 45% and stirred an additional 10 min. The mixture was centrifuged at 10,000 × *g* for 30 min, $(NH_4)_2SO_4$ was added to the supernatant to reach 70%, and the mixture was stirred an additional 10 min. After a final centrifugation at 10,000 × *g* for 30 min, the pellet was collected and dissolved in extraction buffer. Dissolved protein was desalted (Zeba desalt spin columns; Pierce) and quantified (Bradford-based Bio-Rad protein assay reagent).

EPSPS Quantification and Activity Assay. TSP was separated on 12% SDS-polyacrylamide gels. Thirty micrograms of TSP were loaded in each lane for plants with <20 relative *EPSPS* copies, whereas 15 μg TSP were loaded in each lane for plants with >20 relative *EPSPS* copies. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore). Membranes were incubated with primary antibodies against recombinant maize EPSPS at a dilution of 1:2,000. Alexa Fluor 635-labeled goat anti-rabbit secondary antibodies (Invitrogen) were used at a dilution of 1:3,000. Bands were visualized by scanning membranes with a Bio-Rad PharosFX image system equipped with an external 635-nm laser (Bio-Rad), and signals were quantified with Bio-Rad Quantity One software. Membrane signals were normalized according to TSP loading quantity.

A continuous assay for inorganic phosphate release (1) was conducted with a phosphate detection kit (Molecular Probes) to assay for EPSPS activity. The reaction buffer consisted of ultrapure HPLC-grade water, 50 mM Mops, 10 mM $MgCl_2$, 1 mM Na_2MoO_4, 100 mM NaF, 0.2 mM 2-amino, 6-mercapto, 7-methylpurine riboside, 1 U purine-nucleoside phosphorylase, 1.25 mM phosphoenolpyruvate, and glyphosate. Each sample was assayed in triplicate at glyphosate concentrations of 0, 0.1, 1, 5, 10, 25, 50, 100, 500, 1,000, 2,500, and 5,000 μM. This reaction mixture was measured continuously in a spectrophotometer using 5 μg (39 and 54 relative *EPSPS* genomic copies), 25 μg (8 relative *EPSPS* genomic copies), or 50 μg (1 relative *EPSPS* genomic copy) TSP extract. After obtaining a background phosphate release level, the final step was addition of shikimate-3-phosphate to 0.5 mM. Phosphate release was measured for 10 min, and a slope was calculated to determine micromoles of phosphate released per microgram TSP per minute.

1. Webb MR (1992) A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc Natl Acad Sci USA* 89:4884–4887.

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P11043 Petunia x hybrida          97 LGNAGTAMRPLTAAVT 112...306 VKGPPRSSSGRKHLRAIDVNM 326
P23981 Nicotiana tabacum             LGNAGTAMRPLTAAVT     ...     VKGPPRNSSGMKHLRAVDVNM
DQ166525 Fagus sylvatica             LGNAGTAMRPLTAAVT     ...     VTGPPQDSSKKKHLRAIDVNM
FJ869880 A. tuberculatus             LGNAGTAMRPLTAAVA     ...     VTGPPRESSGRKHLRAIDVNM
FJ861242 A. palmeri S                LGNAGTAMRPLTAAVA     ...     VTGPPRDSSGRKHLRAIDVNM
FJ861243 A. palmeri R                LGNAGTAMRPLTAAVA     ...     VTGPPRDSSGKKHLRAIDVNM
                                                  †                          ‡
NM_001063247.1 Oryza sativa          LGNAGTAMRPLTAAVT     ...     VTGPPREPYGKKHLKAVDVNM
DQ153168.2 Lolium multiflorum        LGNAGTAMRPLTAAVV     ...     VTGPPRQPFGRKHLKAVDVNM
AF349754.1 Lolium rigidum            LGNAGTAMRPLTAAVV     ...     VTGPXRQPFGRKHXXAVDVNM
NM_130093.2 Arabidopsis thaliana     LGNAGTAMRPLTAAVT     ...     VTGPPRDAFGMRHLRAIDVNM
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Fig. S1. Glyphosate-resistant *A. palmeri* do not exhibit mutation at Pro106. Alignment of mature plant *EPSPS* amino acid sequences (GenBank accession numbers indicated), including consensus *EPSPS* sequence from seven glyphosate-resistant (R) and two glyphosate-susceptible (S) *A. palmeri* individuals. †No polymorphisms detected in R sequence at Pro106; ‡Polymorphism between R and S *A. palmeri*.

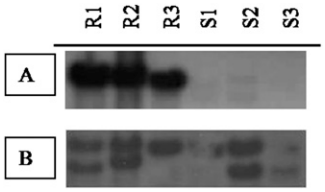

Fig. S2. DNA blot hybridizations suggest that *EPSPS* genomic copy number is higher in glyphosate-resistant than in glyphosate-susceptible *A. palmeri* individuals. Ten micrograms of genomic DNA from three resistant individuals (samples R1–R3) and three susceptible individuals (samples S1–S3) were digested with *DraI*, fractionated on 1% (wt/vol) agarose gels, and transferred to nylon membranes. Blots were then hybridized with a glyphosate-susceptible *A. palmeri* ^{32}P-dCTP labeled *EPSPS* cDNA (*A*) or *ALS* cDNA (*B*), washed at high stringency, and subjected to autoradiography. Similar results were obtained with other restriction enzymes and genomic templates.