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RESEARCH

Isolation of Mutations Conferring Increased Glyphosate Resistance in Spring Wheat

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ABSTRACT

A mutation breeding approach was used to explore the feasibility of isolating glyphosate-resistant (GR) wheat (*Triticum aestivum* L.) lines. Although transgenic GR wheat cultivars were developed, they were never introduced due to lack of consumer acceptance and concern over management of volunteer wheat in rotation. Large-scale screening experiments recovered ethyl methanesulfonate mutants able to resist 360 to 480 g acid equivalent (ae) ha^{-1} glyphosate in four spring wheat cultivars, 'Hollis', 'Louise', 'Macon', and 'Tara2002', indicating that it is possible to recover resistance in a wide range of genetic backgrounds (glyphosate is typically applied at 840 g ae ha^{-1} in transgenic crops). Glyphosate rates of 420 to 530 g ae ha^{-1} were sufficient to kill the susceptible wild-type parents. Seven GR mutants were characterized: GRH9-5, GRH9-8, GRL1, GRL33, GRL65, GRM14, and GRT20. Glyphosate resistance was examined at the whole-plant level in dose–response experiments. Three mutant lines—GRL33, GRH9-5, and GRT20—exhibited resistance based on a significant increase in the dose required to retard growth compared with the corresponding susceptible wild type. According to F_2 segregation analysis, GRL1, GRL65, and GRT20 segregated as a single dominant gene, whereas GRL33, GRH9-5, and GRH9-8 appeared to be either a single semidominant or polygenic trait. Although GRL1 was associated with an amino acid substitution (L239F) in *TaEPSPS-7D1*, no nucleotide changes were observed in the coding regions of wheat *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene in GRL33 and GRH9-8. Results suggest that glyphosate resistance can result from multiple genetic mechanisms in wheat.

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Abbreviations: ae, acid equivalent; AMS, ammonium sulfate; cDNA, complementary DNA; DAT, days after treatment; EMS, ethyl methanesulfonate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; gDNA, genomic DNA; GM, genetically modified; GR, glyphosate-resistant; GR_{50}, the dose required for a 50% growth reduction; mRNA, messenger RNA; NIS, nonionic surfactant; PCR, polymerase chain reaction; RT-qPCR, quantitative reverse transcription polymerase chain reaction; WT, wild type.

Glyphosate [*N*-(phosphonomethyl)glycine] has been widely used for weed control in agricultural systems since 1974. It is a systematic and broad-spectrum herbicide targeting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme required for aromatic amino acid biosynthesis in plants and microorganisms (Cobb and Reade, 2010). Glyphosate is the most commonly used herbicide in weed management (~125,000 t used in 2014 in the United States) and is heavily used in commercial glyphosate-resistant (GR) crop production such as genetically modified (GM) canola (*Brassica napus* L.), cotton (*Gossypium hirsutum* L.), maize (*Zea mays* L.), and soybean [*Glycine max* (L.) Merr.] (Duke and Powles, 2009; Swanson, 2013). Glyphosate is a nonselective herbicide. It can therefore injure sensitive crops such as wheat (*Triticum aestivum* L.) at very low doses, resulting in yield reduction or plant death, often at fractions of the 840-g acid equivalent (ae) ha^{-1} rate, a typical application rate (ae refers to the theoretical yield of parent acid from a pesticide active ingredient that has been formulated as a derivative salt) (Deeds

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et al., 2006). Marketed GR crops, which were modified to carry bacterial gene(s) producing an enzyme that confers resistance to glyphosate, allow growers to use as much as 2 kg ae ha^{-1} glyphosate to control troublesome weeds without damaging crops (Dill, 2005; Pline-Srnic, 2006; Duke et al., 2012). Glyphosate-resistant crop adoption has increased and now comprises >80% of the 120 million ha of transgenic crops grown worldwide (Duke and Powles, 2009). Although transgenic GR wheat was developed (MON71800 in 'Bobwhite'), it is not available for commercial production due to lack of consumer acceptance and concerns over management of escaped or volunteer wheat in fallow and rotational crops (Zhou et al., 1995; Green, 2009; CERA, 2012).

Alternative mechanisms of herbicide resistance in wheat are desirable because weed competition reduces wheat grain yields and can result in inferior grain quality (Stougaard and Xue, 2004). The most significant weeds competing with wheat are grass species common in areas where wheat is the only crop produced, such as areas of the inland Pacific Northwest and portions of the Western and Northern Great Plains. Wild oat (*Avena fatua* L.), feral rye (*Secale cereal* L.), downy brome (*Bromus tectorum* L.), and jointed goatgrass (*Aegilops cylindrica* L.) are troublesome grass weeds with limited available control options. Herbicides are preferable for weed management due to ease of application and effectiveness, being environmentally sound alternatives to cultivation that leaves the soil vulnerable to wind and water erosion. Herbicides facilitate weed management in reduced tillage or direct-seeded cropping systems designed to prevent erosion. Moreover, glyphosate applied at a rate of 840 g ae ha^{-1} to wheat cultivars containing MON71800 (such as GR-Ingot) can control fungal diseases such as stripe rust and leaf rust caused by *Puccinia striiformis* Westend f. sp. *tritici* and *P. triticina* Erikss., respectively (Feng et al., 2005).

Since 1996, several weed species have been discovered with naturally evolved glyphosate resistance as a consequence of selection pressure from continuous heavy use of glyphosate (Powles and Preston, 2006; Heap, 2015). Mechanisms conferring glyphosate resistance in those individuals include mutations of *EPSPS*, overexpression of *EPSPS* transcripts, *EPSPS* gene amplification, reduction of glyphosate translocation (most commonly observed), and reduction of glyphosate retention and absorption (Pline-Srnic, 2006; Michitte et al., 2007; Dinelli et al., 2008; Preston et al., 2009; Gaines et al., 2010). In addition, glyphosate-insensitive *EPSPS* or glyphosate-detoxifying genes derived from microorganisms provide glyphosate resistance in GM crops (Barry et al., 1997; Castle et al., 2004; Dill, 2005). Glyphosate resistance could be associated with one or more mechanisms in the same plant (Powles and Preston, 2006; Perez-Jones et al., 2007). Individuals using more than one mechanism have a greater degree of resistance than those using a single mechanism (Preston et al., 2009). An *EPSPS* mutation resulting in reduced glyphosate translocation should be inherited as a single nuclear gene with complete dominance or semi-dominance—a result observed in weeds with a mutation in *EPSPS* (Preston and Wakelin, 2008; Preston et al., 2009). Similarly, *EPSPS* overexpression, *EPSPS* gene amplification, or decreased glyphosate retention may result in a complete or partially dominant GR phenotype. Amplification of the *EPSPS* gene can also result from effects at multiple loci (Gaines et al., 2011).

Initial work suggested that it is possible to isolate wheat with genetic resistance to herbicides. For example, genetic variation induced by the mutagen sodium azide (NaN_3) was used to develop the imidazolinone-resistant wheat known as 'Clearfield' wheat that is well adopted by the global trade (Tan et al., 2005). Early research suggested that it was not possible to identify induced mutations conferring glyphosate resistance using ethyl methanesulfonate (EMS), a mutagen (Jander et al., 2003). However, an induced P106L mutation (a common point mutation in EPSPS conferring glyphosate resistance) in rice (*Oryza sativa* L.) EPSPS was used to create glyphosate resistance in vitro (Zhou et al., 2006).

The present research focused on the isolation of potential GR spring wheat mutants and the inheritance of glyphosate resistance. The knowledge obtained from this work will serve as a first step to identify the resistance mechanism in wheat mutants for future research. In this study, we (i) screened for potential GR mutants for whole-plant dose response, (ii) studied the mode of gene inheritance in the F_2 populations, and (iii) measured wheat *EPSPS* messenger RNA (mRNA) levels using the quantitative reverse transcription polymerase chain reaction (RT-qPCR) method.

MATERIALS AND METHODS

Germplasm, Mutagenesis, and Retests

Two separate mutagenesis experiments were conducted. The initial mutagenesis experiment was performed in *T. aestivum* spring wheat cultivars soft white 'Louise', hard white 'Macon', and hard red 'Hollis' and 'Tara2002' (Kidwell et al., 2002, 2003, 2004, 2006). M_1 grains were mutagenized with EMS, essentially as in Okubara et al. (2009). In the first smaller-scale experiment, 250 g M_1 grains of Louise, Hollis, and Macon were mutagenized at 0.3% EMS with shaking for 16 h at 22°C. A total of 265,000 M_2 individuals were sprayed with 315 g ae ha^{-1} Roundup ULTRA (0.375×) at the three-leaf stage in the greenhouse.

In the second, large-scale experiment, 2 kg of grain of Louise, Macon, Hollis, and Tara2002 were mutagenized at 0.4% EMS with shaking for 16 h at 22°C. The EMS mutagen was neutralized for 5 min in 5% sodium thiosulfate. Grains were washed 10 times with water and dried before planting. Because M_1 plants are genetic chimeras, they were advanced

from M_1 to M_2 by self-pollination. The M_2 plants were screened for GR mutants, and retests were performed in the M_3 and each advancement of generation (treatment information is described in the herbicide application and scoring condition section below).

The seven GR wheat lines (M_4 or M_5 generation) characterized are listed below, along with original isolation numbers. Lines in the Louise background are: GR Louise1 (GRL1); GR Louise33 (GRL33) (GRL33 originally designated LouiseFR1-33-6-1-4 where 1 is the experiment number [and represents a bulk], 33 is the M_2 isolation number [by single seed selection], 6 the M_3, 1 the M_4, and 4 the M_5); and GR-Louise65 (GRL65) (originally LouiseFR1-65-2-1-1). The Macon mutant GR-Macon14 (GRM14) was originally MaconFR1-14-8-16-1. The Tara2002 mutant GR-Tara20 (GRT20) was originally TaraFR1-20-2-25-2. Original retest experiments characterized multiple isolates derived by single-plant descent. Two isolates of the Hollis mutant GR-Hollis9 (GRH9) were characterized, including GRH9-5 (HollisFR1-9-14-5-3 M_5) and GRH9-8 (HollisFR1-9-14-8 M_4), because they behaved differently in retest experiments.

Plant Growth Conditions and Tissue Sampling

Plants were grown in a greenhouse at 21 to 24°C daytime and 15 to 17°C nighttime temperatures, with a 16-h day and 8-h night photoperiod (Wheat Growth Facility at Washington State University, Pullman, WA). Plants were grown in 10-cm × 10-cm square pots, unless stated otherwise. Young leaves at the four- to five-leaf stage were harvested, immediately submerged in liquid nitrogen, and stored at −80°C until use for nucleic acid extraction. Frozen leaf tissue was ground to a fine powder with a mortar and pestle under liquid nitrogen or with a mechanical mill (Retsch, model MM300).

Herbicide Application and Scoring Conditions

The experiments were conducted in a greenhouse (Wheat Growth Facility, Washington State University) under the conditions as described above. The treatments were composed of glyphosate (Credit Extra, Nufarm, formulated as isopropylamine salt, 360 g ae L^{-1}, or Roundup Ultra, Monsanto, formulated as isopropylamine salt, 356 g ae L^{-1}) at various rates ranging from 0 to 840 g ae ha^{-1} in a solution containing 0.25% v/v of nonionic surfactant (NIS, alkylphenol ethoxylate, butyl alcohol, and dimethylpolysiloxane, R-11) and 24 g L^{-1} of ammonium sulfate (AMS). Water was substituted instead of an active ingredient in the nontreated controls (0 g ae ha^{-1}). Plants were treated at the four- to five-leaf stage. Treatments were applied using an air-pressurized indoor spray chamber equipped with an 80015E flat fan nozzle (Teejet), calibrated to deliver a spray volume of 140 L ha^{-1} at 240 kPa. Visual assessments of plant response were recorded at 14, 21, and 28 d after treatment (DAT). The level of resistance of plants to glyphosate was categorized using a 0-to-4 scale, where 0 was dead (complete mortality, susceptible, S0); 1 was yellowing and stunted but showing small new shoots (resistant, R1); 2 was alive but unhealthy or showing multiple vigorous new shoots (R2); 3 was green but not as healthy as R4 (R3); and 4 was green, healthy, and fertile (R4). Those that were scored as R4 were advanced.

Whole-Plant Dose Response

To evaluate the effect of glyphosate on seven M4 or M5 GR candidates (GRL1, GRL33, GRL65, GRM14, GRH9-5, GRH9-8, and GRT20), a dose response study was conducted. The study was designed as a two-factor factorial with four replications. The first factor was glyphosate dose (0, 110, 210, 420, 630, and 840 g ae ha^{-1}), and the second factor was wheat line (wild type [WT] vs. mutant). The whole study was repeated once in space where a concurrent experiment was conducted in a separate greenhouse. Glyphosate response was quantified by both the visual scoring on the 0-to-4 scale, as indicated above, and by weighing individual aboveground plant biomass harvested at 21 DAT and dried at 70°C. The dried shoot biomass of plants treated with glyphosate was expressed as percentage weight relative to that of the nontreated controls of the same line. A three-parameter log-logistic model described by Seefeldt et al. (1995) was used to calculate the dose required for a 50% growth reduction (GR_{50}) (Eq. [1]). The model was generated using the "drc" package in R (Ritz et al., 2015). Bartlett's test of homogeneity of variances ($\alpha = 0.05$) was used to test if replicated studies could be pooled for analysis (R Development Core Team, 2014). Each cultivar was analyzed separately.

$$y = c + \frac{1-c}{1+\exp^{b[\log(x)-\log(e)]}} \quad [1]$$

where b is the slope at e, c is the lower asymptote, e is the inflection point (GR_{50}), x is the independent variable (rate), and y is the dependent variable (reduction in biomass).

Shikimic Acid Accumulation in Response to Glyphosate in GRL1

A modified spectrophotometric method (Singh and Shaner, 1998; Pline et al., 2002) was used to quantify shikimic acid accumulation in GRL1 and WTL. GRL1 and WTL plants were treated at the three-tiller stage with 420 or 840 g ae ha^{-1} glyphosate, as described in the herbicide application methods section. Nontreated plants were included for comparison purposes. Individual leaves of five replicate plants were harvested at 0, 2, 5, 7, and 9 DAT. The leaf samples were immediately placed in a sealed plastic bag and transported to the laboratory in an iced cooler at 4°C. Extraction techniques followed those of Pline et al. (2002) and Singh and Shaner (1998). Leaf subsamples of GRL1 and WTL plants were harvested using a paper hole punch and placed in 1.5-mL centrifuge tubes and lyophilized. Subsample leaf weights were determined gravimetrically, and then plant material was ground in the microcentrifuge vial using a plastic pestle mounted in a cordless electric drill. After maceration, 0.25 mL of 0.4 M $NaCO_3$ was added, and the extract was agitated and then centrifuged at 10,000*g* for 4 min. The extract was analyzed immediately. Two 40-μL aliquots of each sample were mixed in 0.5 mL of 1% w/v periodic acid in separate 200-μL wells of a 96-well plate and allowed to oxidize. After 3 h, 0.5 mL of 1 M NaOH was added to the sample well and 0.5 mL of deionized water was added to the sample standard well. An additional 0.3 mL of 0.1 M glycine

was added to each vial and agitated. The optical density of each solution was measured at 380 nm. Sample standard values were subtracted from sample values to account for any absorbance caused by plant material, and this standardized value was used to compute the milligrams of shikimic acid per milligram dry weight of onion (*Allium cepa* L.) leaf based on a standard curve (Singh and Shaner, 1998; Pline et al., 2002). Standard curves were developed by using pure shikimic acid standard of known concentrations. The experiment was repeated in time.

Development of Backcross Populations

Plants were grown in 16.5-cm-diam. ×18-cm-depth round pots supplemented once after planting with 14–14–14 fertilizer (Osmocote). Each GR mutant of the seven candidates was crossed to its corresponding WT of the same cultivar to generate the BC_1F_1 generation. The GR mutant parent was used as a female, whereas the WT parent was used as a male. Plants were covered with transparent nylon bags to prevent pollen contamination. The BC_1F_1 plant was self-pollinated to yield BC_1F_2 populations for use in gene segregation analyses.

Segregation Analysis

The BC_1F_2 populations from single F_1 plants (200–250 individuals) for each of the seven GR candidates were grown until the first tiller appeared. Each individual plant was dissected into two clones (carrying identical genetic material), transplanted separately, and allowed to recover for 2 to 3 wk. Eight plants each of the WT and GR parents and the BC_1F_2 plants were treated with 420 g ae ha^{-1} glyphosate. For each BC_1F_2 plant, one clone was left nontreated and one was glyphosate treated and scored for glyphosate resistance using a 0-to-4 scale at 28 DAT. Chi-square analysis was used to test goodness-of-fit to either a recessive or dominant mode of inheritance at a significance level of 0.05 ($\chi^2 < 3.84$, df = 1, or $\chi^2 < 5.99$, df = 2). If the observed χ^2 value was less than the criteria χ^2 value ($p > 0.05$), the observed segregation ratio statistically fit the expected ratio.

Nucleic Acid Isolation and cDNA Synthesis

Genomic DNA (gDNA) and total RNA were isolated from 100 and 50 mg of ground leaf tissue, respectively. The gDNA of hexaploid wheat Hollis was isolated using BioSprint DNA Plant 96 extraction kit (Qiagen) following the manufacturer's procedure. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Before performing RT-qPCR, RNA samples were treated with 2 U of DNase I to prevent DNA contamination and passed through DNA-free RNA kit (Zymo Research). Nucleic acid concentration and quality were assessed by NanoDrop ultraviolet spectrophotometry and by agarose gel electrophoresis. For complementary DNA (cDNA) cloning experiments, first-strand cDNA was synthesized from 5 μg of total RNA using the ImProm-II reverse transcription system kit (Promega). For RT-qPCR experiments, first-strand cDNA was synthesized from 1 μg of total RNA using the ProtoScript first strand cDNA synthesis kit (NEB). Synthesized cDNA was adjusted to a final concentration of 100 ng μL^{-1} for cDNA cloning and to 2 ng μL^{-1} with RNase-free water for RT-qPCR.

PCR, Cloning, and Sequencing

The polymerase chain reaction (PCR) amplification conditions were identical for every primer pair, except for annealing temperature and extension time, provided in Supplemental Table S1. The PCR products were visualized on a 1% agarose gel stained with SYBR Safe (Invitrogen). The DNA fragments were excised from the gel, purified using the QIAEX II gel extraction kit (Qiagen), and cloned into the pGEM-T easy vector (Promega). Ligation reactions were transformed into *Escherichia coli* JM109, and bacterial colonies carrying recombinant plasmids with the target insert were selected by blue-white screening and confirmed by PCR. Plasmid DNA was purified using the QIAprep miniprep kit (Qiagen) and sequenced by Elim Biopharmaceuticals (Hayward, CA) or the Molecular Biology and Genomics Core (Washington State University, Pullman).

Cloning of Three *TaEPSPS* Sequences

Primers used in the *EPSPS* cloning were developed based on the DNA sequences of *TaEPSPS-7A1* (KP411547), *TaEPSPS-7D1* (KP411548), and *TaEPSPS-4A1* (KP411549) of the WT Louise wheat cultivar (Aramrak et al., 2015; Supplemental Tables S1 and S2). GRL1 and GRL33 cDNA was used as a template to amplify Exons 2 through 8 with the F3/R1 primer pair (1190 bp of the 1533-bp *EPSPS* mRNA sequence). It was not possible to clone the *EPSPS* cDNA sequence 5′ of F3 because the sequence is highly GC-rich. However, most (216 bp) of the upstream sequence is cleaved on import into the chloroplast. The PCR amplification was performed in 15 μL of total PCR reaction containing 200 ng of cDNA, 0.2 mM deoxynucleotides, 2 mM $MgCl_2$, 0.4 μM of F3 and R1 primers, 0.5 U proofreading *LA Taq* DNA polymerase (TaKaRa), and 1× Mango buffer (Bioline). The F3/R1 primer pair was expected to amplify all three cDNA *EPSPS* copies. Instead, they amplified *TaEPSPS-7A1* and *TaEPSPS-7D1*, but not *TaEPSPS-4A1*. Therefore, genome-specific primer Int1_F3-4A was combined with a common primer TaEPSPS_R and used to clone the genomic copy of *TaEPSPS-4A1* from GRL1 and GRL33 (1068 of 3342 bp, including Exons 2–4). The genomic copies of all three *EPSPS* genes of WT Hollis, GRH9-5, and GRH9-8 were cloned by PCR amplification using the genome-specific primers Int1_F3-7A, Int1_F3-7D, and Int1_F3-4A combined with a common primer TaEPSPS_R. The PCR reaction (total volume 15 μL) was performed with 50 ng of gDNA, 0.4 μM of each primer, and 1× GoTaq Premix (Promega). Sequence was obtained from 12 cDNA and four independent gDNA clones.

Quantitative RT-qPCR Analysis

The RT-qPCR analysis examined *EPSPS* transcript levels in wheat plants treated with or without glyphosate. Nine plants of both WT Louise and GRL1 at the four- to five-leaf stage were treated with glyphosate at the rates of 0 (nontreated control) or 530 g ae ha^{-1}. The nontreated control was sprayed with water, 0.25% v/v NIS, and 24 g L^{-1} AMS. Seedling tissue of each individual plant was harvested before the application [sample size (n) = 6 for basal levels] and 1 h after application (n = 3 for 1 h post-nontreated control and n = 3 for 1 h after glyphosate treatment). At 14 DAT, four GRL1 plants survived the treatment, whereas all WT Louise plants died. Thus, tissue samples

from three biological replicates of susceptible WT Louise and of three resistant GRL1 plants were used to study expression levels of *EPSPS* genes. Three biological replicates of each line from the nontreated control were also used in the analysis as controls.

Two-step RT-qPCR analysis of the *EPSPS* transcripts of *TaEPSPS-7A1*, *TaEPSPS-7D1*, and *TaEPSPS-4A1* was performed using genome-specific primers (as in Aramrak et al., 2015; Supplemental Tables S1 and S2). Complementary DNA was generated using a poly-T primer and the ProtoScript first-strand cDNA synthesis kit (NEB). One microliter of the cDNA reaction was used as a template for quantitative PCR amplification performed using the LightCycler FastStart DNA Master SYBR Green I Kit and the LightCycler carousel-based system (Roche) (Aramrak et al., 2015). Total RNA without reverse transcription (no RT) served as a negative control to screen for gDNA contamination. No template controls, where nuclease-free water was substituted for cDNA, were used to screen for possible contamination. Two technical replications were performed for each sample of each primer pair.

The wheat cyclophilin (*TaCP*) gene was used for normalization of each *TaEPSPS* gene copy (Paolacci et al., 2009). A twofold serial dilution of gDNA or cDNA samples was used to construct a standard curve to determine amplification efficiency (98.8–103.5%). Melting curves were generated, ranging from 70 to 96°C after amplification, to assess the specificity of amplified PCR products. The relative fold change was determined using the comparative delta delta method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

Data Analysis

Sequence alignments were performed using ClustalW (Larkin et al., 2007) and Geneious software (Kearse et al., 2012). The protein structure of the putative mutated TaEPSPS-7D1 was computed based on the crystal structure of *E. coli* EPSPS in complex with shikimate and glyphosate (obtained from Protein Data Bank, 2aay file) using Swiss-Pdb Viewer (SPDBV) software (Guex and Peitsch, 1997). Statistical significance was determined by ANOVA using a Tukey's comparison at the significance level of 0.05 in SAS version 9.3 (SAS Institute, 2011). Probability values ≤ 0.05 were considered statistically significant. For RT-qPCR analysis, transcript fold changes were $\log_{10}$ transformed to stabilize distribution and variance homogeneity. The figure illustrating fold change shows $\log_{10} + 1$ transformed values to make all values positive.

RESULTS

Mutant Isolation

Because it was unclear if glyphosate resistance could be selected using forward genetics in wheat, a preliminary screen using treatment with low glyphosate rates was performed on 0.3% EMS-mutagenized Louise, Macon, and Hollis cultivars. Four M_2 candidate plants (two Louise, one Hollis, and one Macon) survived. Of these, only one candidate (GRL1) survived the M_3 retest consisting of two applications of 158 g ae ha^{-1} glyphosate, for a total application of 315 g ae ha^{-1}.

As the results of the first small-scale experiment indicated that it was possible to identify EMS-induced mutations resulting in increased survival of glyphosate treatment, a large-scale mutant screen was conducted in the field. The spring wheat cultivars Louise, Hollis, Tara2002, and Macon were mutagenized with 0.4% EMS and advanced from M_1 to M_2 in the field. One and a half million M_2 plants were sprayed twice (1 wk apart) with 240 g ae ha^{-1} Roundup ULTRA, for a total application rate of 480 g ae ha^{-1}. The approach of using two applications was intended to decrease the likelihood of recovering plants that escaped the first glyphosate field treatment. One hundred and fifty-seven M_2 plants survived this field screen and were advanced to the M_3. Of the 157 $M_{2:3}$ lines, 33 M_3 lines survived greenhouse retest experiments at a 315-g ae ha^{-1} application rate, and 10 of these survived a field test at 420 g ae ha^{-1} Roundup ULTRA. Of the original 33 lines that survived the M_3 retest, six isolates were selected for further detailed analysis based on plant health and survival in the M_4 and M_5 retest experiments (Supplemental Table S3). Plants used in retests were derived by single-plant descent to allow selection of lines that no longer segregated for glyphosate resistance. Because there was concern that GRH9 might have still been segregating in the M_5 retests, two families derived from GRH9 (GRH9-5 M_5 and GRH9-8 M_4) were used for glyphosate dose–response and segregation analyses.

Whole-Plant Dose Response of Resistant and Susceptible Lines

Seven mutant lines (GRL1, GRL33, GRL65, GRM14, GRT20, GRH9-5, and GRH9–8), plus the corresponding WT parents Louise (WTL), Macon (WTM), Tara2002 (WTT), and Hollis (WTH), were evaluated for glyphosate tolerance in a dose–response assay. The response to glyphosate was measured based on whole-plant aboveground biomass (g) in treated versus nontreated samples for each genotype. The glyphosate rate of 210 g ae ha^{-1} resulted in a greater decrease in WT than mutant biomass ($p < 0.05$) in all comparisons, except for WT versus GRH9-8, GRM14, GRL1, or GRL65 (Fig. 1). The glyphosate rate of 420 g ae ha^{-1} revealed the clearest differences between WT and mutants, resulting in approximately an 80% decrease in WT biomass. Therefore, the 420-g ae ha^{-1} rate of glyphosate was selected for use in subsequent experiments.

The GR_{50} values were used to determine which mutants resulted in a significant increase in glyphosate tolerance compared with the corresponding WT (Table 1). Since Bartlett's test of homogeneity did not indicate differences in variance between the two study replicates, the results were combined for analysis. The dose–response curves were generated using a log-logistic model, where GR_{50} for each line was obtained from the analyses (Fig. 1, Table 1). The M_5 isolate GRH9-5 expressed increased

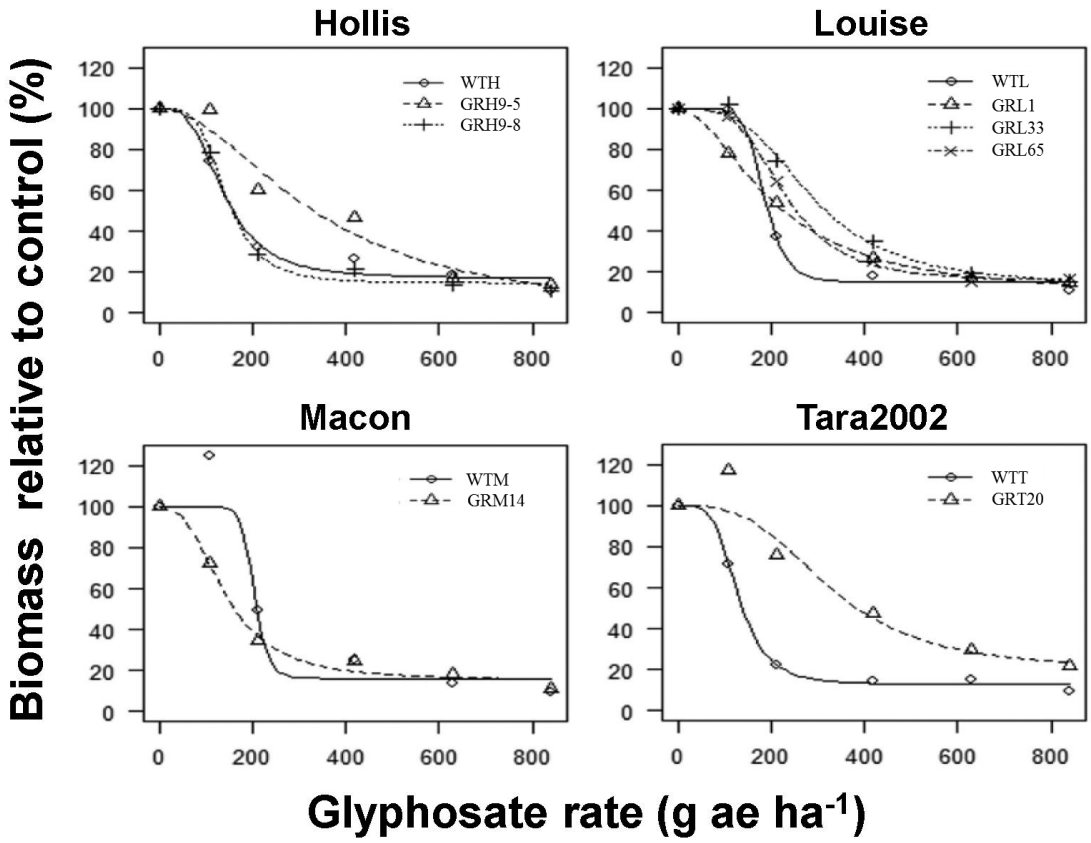

Fig. 1. Dose-dependent response to glyphosate at the level of whole-plant biomass. The *y*-axis shows the dry aerial biomass (g) of the treated plants, expressed as a percentage of the nontreated control biomass for each genotype. The average dry biomass of eight plants per genotype was used in the calculation. The *x*-axis indicates the dose of glyphosate (g acid equivalent [ae] ha^{-1}). A *t* test was used to examine whether there was a significant difference between wild type (WT) and mutant at 210 g ae ha^{-1}.

glyphosate resistance (2.4-fold) compared with WT Hollis, whereas the other M_5 isolate, GRH9-8, did not. Thus, care will need to be taken to identify nonsegregating isolates of GRH9. Among the Louise GR lines, GRL33 expressed stronger glyphosate resistance than GRL1 and GRL65, resulting in a 1.5-fold increase in GR_{50} compared with WT Louise. Line GRT20 showed a significant 2.5-fold increase in GR_{50} compared with WT Tara2002. The GR_{50} dose for GRM14 was significantly lower than that of the corresponding WT Macon, indicating that GRM14 was more susceptible to glyphosate than WT. Hormesis was observed for WTH, where plant biomass was greater with the lowest dose of glyphosate than with no treatment. GRM14 also has very different plant architecture compared with WT Macon (Supplemental Fig. S1). Nontreated GRM14 plants were shorter but bushier than WT. Upon glyphosate treatment, GRM14 plants were stunted but remained green, and the nontreated were less vigorous than WTH. The green color is the reason GRM14 was selected in the screen for GRt plants. Alternatively, GRM14-independent background mutations from the EMS mutagenesis may be reducing the vigor of GRM14.

Table 1. Glyphosate dose required for 50% plant growth reduction (GR_{50}).

Cultivar	Line	GR_{50} (± SE)	Mean difference†
Hollis	WTH	139.06 (± 11.09)	a
	GRH9-8	143.80 (± 9.77)	a
	GRH9-5	334.85 (± 77.08)	b
Louise	WTL	185.10 (± 19.70)	a
	GRL1	207.26 (± 36.81)	ab
	GRL65	230.24 (± 19.98)	ab
	GRL33	287.63 (± 32.95)	b
Macon	WTM	203.46 (± 8.42)	a
	GRM14	141.28 (± 15.93)	b
Tara2002	WTT	129.77 (± 9.26)	a
	GRT20	327.50 (± 46.99)	b

† Same letter indicates that there is no statistically significant difference using a log-likelihood ratio test in the analysis.

The GRL mutants were found to have varying degrees of resistance to glyphosate, suggesting that they may result from different mechanisms for glyphosate resistance. GRH9-5 and GRH9-8 were derived from the same mother M_3 plant. Our observations indicated that the GRH9-5 isolate has greater glyphosate resistance than GRH9-8. It may be that either the mutations or detrimental background mutations are still segregating in the M_5 and M_6 generations.

Shikimic Acid Accumulation in Response to Glyphosate in GRL1

Shikimic acid accumulation was different between GRL1 and WTL (Fig. 2). In GRL, shikimic acid accumulation different from the nontreated was only observed at 7 DAT. Increased shikimic acid accumulation was observed at 9 DAT in GRL1 and may be indicative of inhibition of sensitive EPSPS or a natural increase in shikimic acid pools within the plant. Shikimic acid accumulated in WTL and was different from the nontreated at each harvest interval, except for 0 DAT. At 9 DAT, shikimic acid accumulation in response to 840 g ae ha^{-1} glyphosate was lower than with no treatment.

Inheritance of Glyphosate Resistance

BC_1F_2 populations derived from crosses between the GR mutants and the corresponding WTs were used to investigate the genetic segregation of glyphosate resistance. The susceptible and resistant phenotypes were scored based on apparent survival at 28 DAT (see Materials and Methods). The χ^2 test for goodness-of-fit ($\alpha = 0.05$) was used to determine whether the segregation ratio was consistent with inheritance as a single recessive or single dominant trait (Table 2). The BC_1F_2 populations derived from GRM14 fit a segregation ratio of 1:3 resistant:susceptible ($\chi^2 = 0.024$, df = 1), suggesting that glyphosate resistance resulted from a single recessive gene. However, given that GRM14 showed weak glyphosate resistance in the dose–response experiment (Table 1), this result must be interpreted with caution. It is possible that the apparent segregation as a single recessive gene is due to the low penetrance of glyphosate resistance in GRM14. In addition, the GRM14 mutant exhibited a dwarf phenotype. The dwarf phenotype fit a segregation ratio of 3:1 tall:short ($\chi^2 = 0.075$, df = 1), suggesting that a single recessive gene is responsible for the dwarf trait in the GRM14 mutant. However, the dwarfism did not always cosegregate with glyphosate resistance.

The BC_1F_2 populations derived from GRL1, GRL65, and GRT20 fit a segregation ratio of 3:1 resistant:susceptible ($\chi^2 = 2.47$, 1.65, and 1.56, df = 1, respectively), indicating that glyphosate resistance results from a single dominant gene. The GRT20 mutant also exhibited a club head phenotype, whereas WT had a lax head. The segregation ratio of head types was observed with 1:2:1 lax head:intermediate:club head ($\chi^2 = 5.41$, df = 2), suggesting that a single semidominant gene is responsible for the head type trait (Tingey, 1924). The BC_1F_2 populations derived from GRL33, GRH9-5, and GRH9-8 failed to fit the segregation ratio for either a single recessive or dominant trait, suggesting that a semidominant or multiple-gene trait may be responsible for the segregation of glyphosate resistance. The highest degree of glyphosate resistance was observed in GRH9-5 and GRT20 (Table 1. Supplemental Fig. S2). Thus, it is not the case that the dominant phenotype always coincides with the strongest glyphosate resistance phenotype.

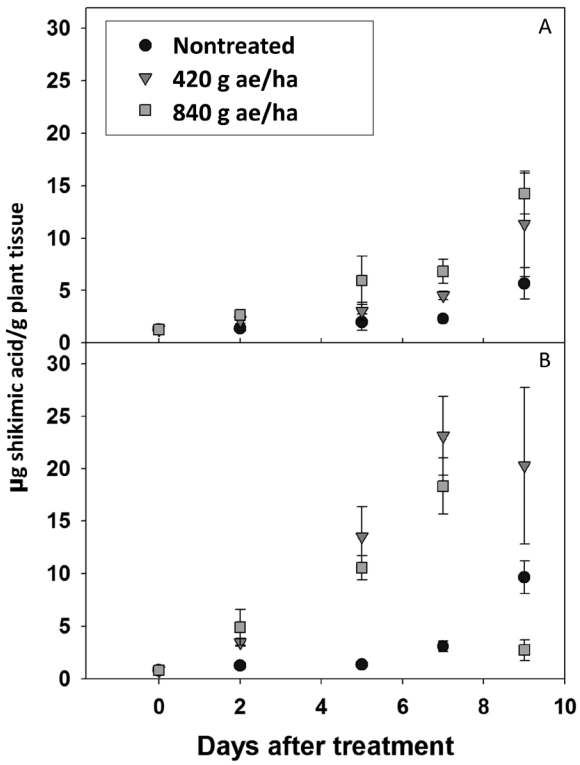

Fig. 2. Shikimic acid accumulation in (A) GRL1 and (B) Louise over 9 d after treatment with or without the indicated concentration of glyphosate. Error bars indicate standard error of the mean where *n* = 10. ae, acid equivalent.

TaEPSPS mRNA Expression Levels in GRL1

Glyphosate resistance can result from increased mRNA expression of *EPSPS* due either to increased *EPSPS* copy number or regulatory mutations (Gaines et al., 2010). Thus, we examined whether GRL1 resulted in increased *TaEPSPS-7A1*, *TaEPSPS-7D1*, or *TaEPSPS-4A1* mRNA levels in GRL1 relative to WT Louise by two-step RT-qPCR analysis (Fig. 3). Previous work suggested that *EPSPS* gene expression may increase within a short time after glyphosate treatment (Gaines et al., 2011). Thus, expression in WT and GRL1 was examined both without and after glyphosate treatment. No significant differences in the three *TaEPSPS* transcript levels were observed between WT Louise and GRL1 either without or 1 h after glyphosate treatment. However, there was a significant increase when the basal level (no treatment) was compared with the glyphosate treatment for *TaEPSPS-7A1* and *TaEPSPS-7D1*. The control treatment appears to affect the expression of wheat *EPSPS*, especially of *TaEPSPS-7D1*. Thus, it appears that treatment with either the control or glyphosate may cause an increase in *EPSPS* mRNA levels 1 h after treatment. The 1-h time point was chosen because we wanted to avoid comparing gene expression in living and dying plants.

Table 2. Segregation analysis for glyphosate resistance in BC_1F_2 populations.

BC_1F_2 population	Parental cross	Observed		Total plants	Expected		Expected ratio	χ^2 (0.05, df = 1)	*p*-value	Mode of inheritance
		R†	S		R	S				
Hollis										
GRH9-8	GRH9-8 × WTH	82	152	234	58.5	175.5	1:3	12.57	0.0004	Semidominant or QTL‡
GRH9-5	GRH9-5 × WTH	130	95	225	112.5	112.5	1:1	29.94	<0.0001	Semidominant or QTL
Louise										
GRL1	GRL1 × WTL	160	67	227	170.25	56.75	3:1	2.47§	0.12§	Single dominant gene
GRL65	GRL65 × WTL	184	50	234	175.5	58.5	3:1	1.65§	0.20§	Single dominant gene
GRL33	GRL33 × WTL	206	40	246	184.5	61.5	3:1	10.02	0.0015	Semidominant or QTL
Macon										
GRM14	GRM14 × WTM	57	167	224	56	168	1:3	0.024§	0.78§	Single recessive gene
Tara2002										
GRT20	GRT20 × WTT	183	50	233	174.75	58.25	3:1	1.56§	0.21§	Single dominant gene

† R, resistant; S, susceptible.

‡ QTL, quantitative trait locus.

§ Statistically fit the expected ratio ($\chi^2 \leq 3.84$, df = 1 or *p*-value ≥ 0.05).

TaEPSPS Sequence Analysis in GRL1, GRL33, and GRH9 Mutants

Wheat *EPSPS* sequences of *TaEPSPS-7A1* (KP411547), *TaEPSPS-7D1* (KP411548), and *TaEPSPS-4A1* (KP411549) of GRL1, GRL33, GRH9-5, and GRH9-8 lines were examined for EMS-induced point mutations. Mutations altering intron splicing junctions of *EPSPS* would more likely result in abnormal EPSPS and thus decreased, rather than increased, glyphosate resistance. Thus, we chose to focus on cloning cDNA sequence from each of the three wheat *EPSPS* genes. Previous work showed that mutations in the proline residue (P106 to L, S, T, or A) of the conserved "LFLGNAGTAMPL" motif of EPSPS resulted in GR weed biotypes (Baerson et al., 2002b; Perez-Jones et al., 2007; Kaundun et al., 2011). This P106 corresponds to P172 of wheat EPSPS *TaEPSPS-7A1* (KP411547). Since EMS induces GC to AT transitions, it could cause either a Pro172 to Ser or Leu substitution in wheat EPSPS (Greene et al., 2003). Thus, all of the primer pairs used for mutant cloning and sequencing included the sequences encoding Pro172.

The F3/R1 primer pair was used to obtain 1190-bp *EPSPS* cDNA clones from GRL1 and GRL33 mutants (Tables 1 and 2). Twelve independent clones of each mutant Louise line were randomly selected to sequence. Multiple 1190-bp clones were recovered for *TaEPSPS-7A1* and *TaEPSPS-7D1*, but no copies of *TaEPSPS-4A1* were recovered due to the fact that this transcript is expressed at lower levels (Aramrak et al., 2015). Therefore, *TaEPSPS-4A1*-specific primers (Int1_F3-4A and TaEPSPS_R) were used to recover 1068-bp genomic clones of *TaEPSPS-4A1* from GRL1 and GRL33 (Tables 1 and 2). The expected *TaEPSPS-4A1* cDNA sequences were derived from the genomic clones and used for alignment with the WT Louise sequence (GRL1_4A-C# and GRL33_4A-C#) (Fig. 4). The *EPSPS* cDNA sequences were aligned and examined for a potential mutation compared with the previously

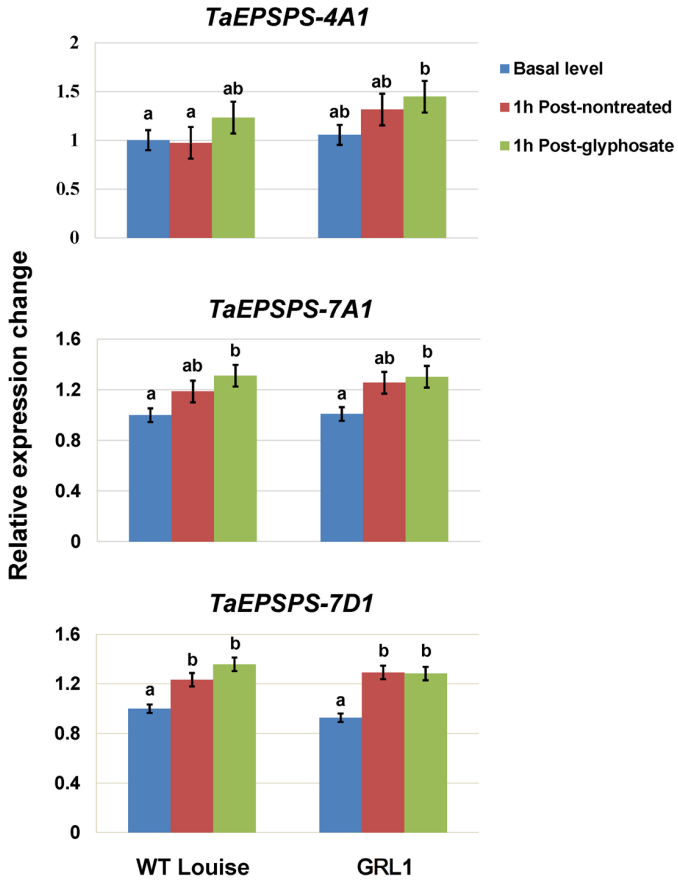

Fig. 3. Expression analysis of the wheat *EPSPS* homoeologous genes. The expression levels of *TaEPSPS-7A1*, *TaEPSPS-7D1*, and *TaEPSPS-4A1* were examined in WTL and GRL1 lines under the following conditions: (i) prior to treatment (basal level, blue), (ii) after treatment with nonionic surfactant (NIS) and ammonium sulfate (AMS) without glyphosate (1 h post-nontreated, red), and (iii) after treatment with 530 g acid equivalent ha^{-1} glyphosate in a solution containing NIS and AMS (1 h post-glyphosate, green). The *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) fold change was calculated relative to the expression at a basal level of nontreated WTL. Bars indicate standard error of the mean, and letters represent significant differences ($P < 0.05$, based on Tukey's pairwise comparison at a 95% confident interval).

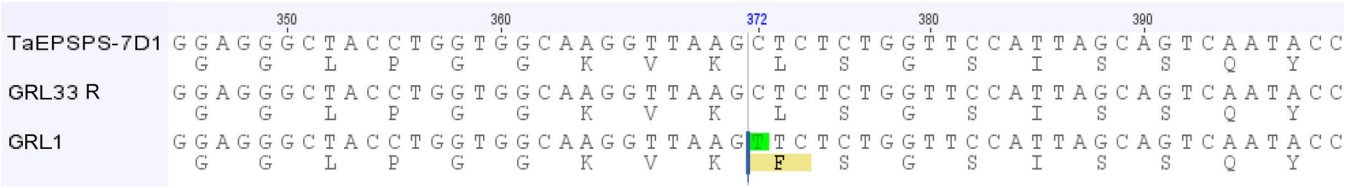

Fig. 4. Nucleotide alignment of *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) complementary DNA (cDNA) sequences from wheat 'Louise' lines. The cDNA sequences were obtained from the cDNA clones (GRL#_C#) and derived from the 4A-genomic DNA clones (GRL#_4A-C#) of *T. aestivum* GRL1 and GRL33 mutant lines. The cDNA sequences derived from the genomic DNA clones of *T. aestivum* Louise wild-type are shown for comparison and labeled TaEPSPS-7A1, TaEPSPS-7D1, and TaEPSPS-4A1. Highlighting indicates the point mutation caused by ethyl methanesulfonate induction, resulting in the nucleotide alteration from C to T at position 715 in *TaEPSPS-7D1* of four GRL1 clones. Labels 7A, 7D, and 4A represent clusters of *EPSPS* genes.

reported *TaEPSPS-7A1* (A genome), *TaEPSPS-4A1* (B genome), and *TaEPSPS-7D1* (D genome) of WT Louise (Fig. 4, Supplemental Fig. S3). No mutations were observed in the three GRL33 *EPSPS* genes, including the conserved "P106." No mutations were observed in GRL1 *TaEPSPS-7A1* and *TaEPSPS-4A1* cDNA sequences. However, the *TaEPSPS-7D1* gene of the GRL1 mutant contained a nucleotide change from CTC to TTC at position 715 (C715T), resulting in a predicted amino acid substitution of Phe instead of Leu at position 239 (L239F) (Fig. 5).

Genomic copies of *TaEPSPS-7A1*, *TaEPSPS-7D1*, and *TaEPSPS-4A1* were cloned from GRH9-5, GRH9-8, and WT Hollis using genome-specific primers Int1_F3-4A, -7A, and -7D with the reverse primer TaEPSPS_R. Four independent clones from each line were sequenced for each *EPSPS* gene. The deduced cDNA sequences derived from the genomic DNA clones of *TaEPSPS-7A1* (1119 bp), *TaEPSPS-7D1* (1015 bp), and *TaEPSPS-4A1* (1068 bp) were used in alignments. No point mutations were detected in the three *TaEPSPS* genes of GRH9-5 and GRH9-8 compared with WT Hollis (Supplemental Fig. S4). Thus, the glyphosate resistance phenotypes of these two mutants do not appear to result from a missense mutation in the *EPSPS* coding region.

DISCUSSION

Level of Glyphosate Resistance

The research presented here demonstrates that it is possible to generate nontransgenic wheat plants with increased glyphosate resistance using a mutation breeding approach. However, based on the GR_{50} values, the degree of glyphosate resistance (141 to 335 g ae ha^{-1}, Table 1) in induced GR wheat mutants is lower than that of the naturally evolved glyphosate-resistant weed biotypes. For example,

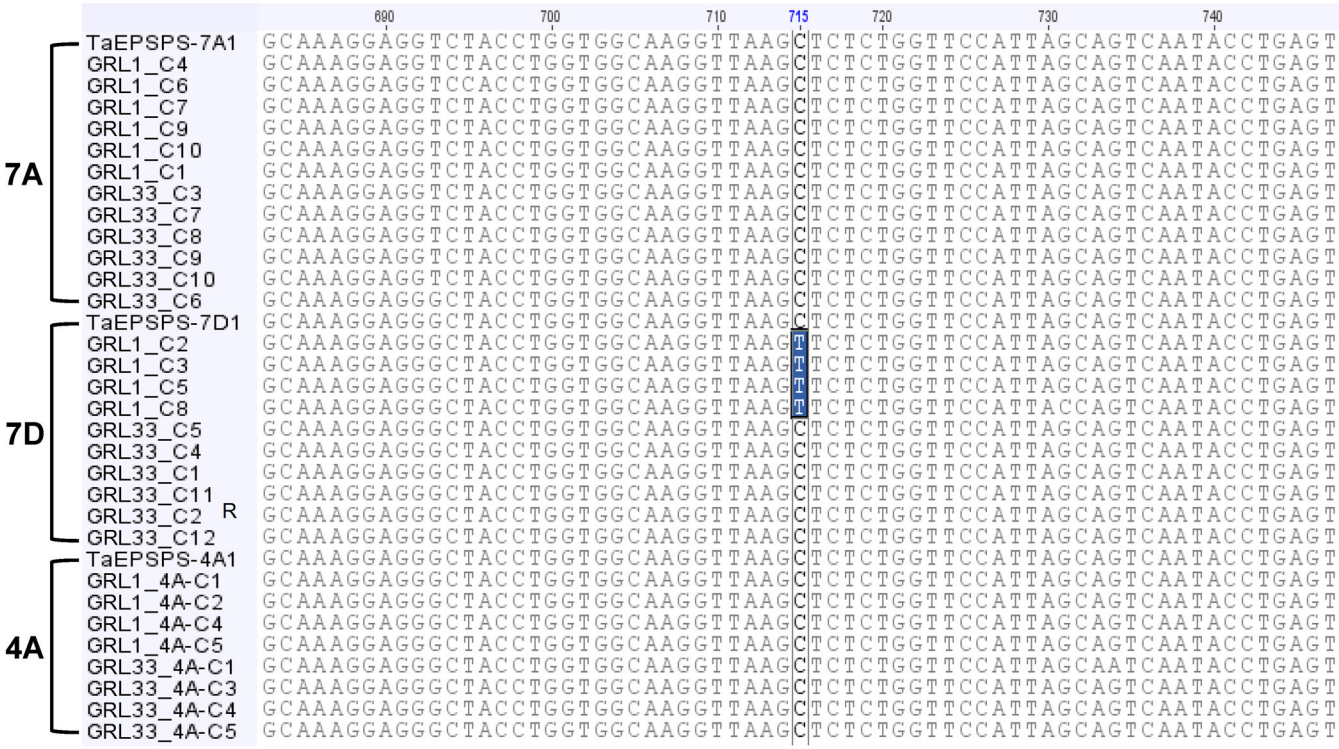

Fig. 5. Ethyl methanesulfonate-induced point mutation on the TaEPSPS-7D1 of GRL1 mutant. The amino acid residues were translated from *TaEPSPS-7D1* complementary DNA sequences. The mutation of C715T (red arrow) results in amino acid substitution of L239F in TaEPSPS-7D1 of four GRL1 clones. Highlighting indicate the point mutations.

the GR_{50} dose for the strongest wheat mutant (GRH9-5) is 335 g ae ha^{-1}, whereas naturally occurring GR weed biotypes showed a GR_{50} of 465 to 514 g ae ha^{-1} in *Lolium rigidum* (L.) Gaud, of 461 to 868 g ae ha^{-1} in *Conyza bonariensis* (L.) Cronquist, and of 945 to 1596 g ae ha^{-1} in *L. perenne* ssp. *multiflorum* (Lam.) (Lorraine-Colwill et al., 2001; Dinelli et al., 2008; Salas et al., 2012). Thus, it seems unlikely that naturally occurring single gene resistance could result in GR wheat lines arising spontaneously in farmers' fields.

There are two possible explanations for the low level of glyphosate resistance in GR mutants: (i) the allohexaploid nature of wheat may prevent the full expression of glyphosate resistance phenotypes from single-gene mutations, or (ii) background mutations resulting from mutagenesis may reduce the overall vigor of GR wheat. It may be necessary to combine multiple resistance mechanisms to obtain commercially useful levels of glyphosate resistance in developing GR wheat. Once the genes altered in GR wheat lines have been identified, it should be possible to derive wheat plant with improved resistance using gene targeting approaches (Carroll, 2014). Both naturally occurring and engineered glyphosate resistance have made use of multiple glyphosate resistance mechanisms (Dill, 2005; Pline-Srnic, 2006; Duke et al., 2012). Selection pressure from low doses of herbicide can result in increasing numbers of glyphosate resistance genes over time. Thus, the tactic of combining multiple glyphosate resistance mechanisms is the most promising way to develop non-GM GR wheat cultivars (Preston et al., 2009).

The Genetics of Glyphosate Resistance

The segregation analysis of the GRH9-5 and GRH9-8 lines raised the possibility that more than one gene may be affecting glyphosate resistance in descendants of the original GRH9 M_3 mutant. The χ^2 analysis of F_2 segregation data showed that both GRH9-5 and GRH9-8 failed to fit the expected segregation of a single recessive or dominant trait (Table 2). However, the two lines behaved differently in F_2 segregation analyses. Of the GRH9-8 F_2s, 35% survived glyphosate application, whereas 58% of the GRH9-5 F_2s survived. The 35% value is not low enough for a recessive trait (expected 25% survival), and 58% is not high enough to be considered a dominant trait (expected 75% survival). The discrepancy between the two descendants of GRH9 caused us to hypothesize that the phenotype was being influenced by other mutations. Future work will need to take into consideration the possibility that GR lines segregate for negative alleles that may have epistatic interactions with the gene causing glyphosate resistance (Mackay, 2014; Stirnweis et al., 2014).

The differences between the GRH9-5 and GRH9-8 lines derived from the same mutant emphasize the importance of backcrossing lines derived from EMS mutagenesis. Ethyl methanesulfonate can induce mutations at frequencies as high as one mutation in 24 kb in hexaploid wheat, giving ~708,000 mutations in the 17-Gbp wheat genome (Slade et al., 2005). It is possible that EMS-induced mutations enhance or decrease metabolic activities that affect glyphosate response in the plant (Al-Qurainy and Khan, 2009). For example, these mutations may increase the need for EPSPS by altering aromatic amino acid biosynthesis, uptake, or transport. The populations used in this study were derived either directly from mutagenesis or from the first backcross to WT. Therefore, crosses to a WT plant that do not contain such background mutations are essential to fully understand the mode and degree of herbicide resistance.

Transcription Levels of Wheat *EPSPS*

Previous work showed that glyphosate resistance could result from increased *EPSPS* mRNA expression levels from (i) a single *EPSPS* copy in *Lolium rigidum* Gaud., *Dicliptera chinensis* Juss., and *Conyza bonariensis* L., and (ii) multiple copies of *EPSPS* in *Amaranthus palmari* L. and *L. multiflorum* L. (Baerson et al., 2002a; Yuan et al., 2002; Dinelli et al., 2008; Gaines et al., 2010; Salas et al., 2012). We compared GRL1 and WT Louise *TaEPSPS* expression levels before and 1 h after treatment with glyphosate or a mock control (Fig. 3). *EPSPS1* transcript levels increased within 1 h of glyphosate treatment in GRt *Glycine soja* Sieb. & Zucc (ZYD-254 line) and in *Ageratum houstonianum* Mill. (a susceptible biotype of *D. chinensis*) (Yuan et al., 2002; Gao et al., 2014). Moreover, GR *C. bonariensis* and *L. rigidum* biotypes had significantly higher *EPSPS* mRNA levels than susceptible biotypes without glyphosate treatment (Baerson et al., 2002a; Dinelli et al., 2008). Thus, it was expected that *TaEPSPS* transcript levels might be higher at either the basal or 1-h time point. However, no differences were observed in the three *TaEPSPS* gene transcript levels of GRL1 and WT Louise, suggesting that GRL1 glyphosate resistance does not result from *EPSPS* mRNA overaccumulation (Fig. 3).

The observation that there was no statistically significant increase in *EPSPS* mRNA levels in wheat 1 h after glyphosate application was not inconsistent with observations in other species. Glyphosate-resistant biotypes of *Conyza canadensis* L. and *Lolium rigidum* with non-target-site resistance did not express a significant increase in *EPSPS* mRNA levels within 24 h after application (Yu et al., 2009; Nol et al., 2012). Another study in WT *Nicotiana* demonstrated that *EPSPS* expression levels did not change within 1 to 3 DAT, possibly because the remaining aromatic amino acid pools can support the plant. The *EPSPS* mRNA levels increased at 6 to 14 DAT to replenish the depleted aromatic amino acid stores and declined after 14 DAT as the plant died (Garg et al., 2014). Future work can examine if something similar may be occurring in the regulation of wheat *EPSPS* expression.

Mechanisms of Glyphosate Resistance

Based on previous research, we can speculate as to what types of mutations may result in a glyphosate resistance phenotype. Naturally occurring genetic variation has conferred glyphosate resistance as a result of (i) amino acid changes in EPSPS, (ii) increased expression of *EPSPS*, and (iii) decreased glyphosate translocation or absorption (Pline-Srnic, 2006; Michitte et al., 2007; Dinelli et al., 2008; Preston and Wakelin, 2008; Preston et al., 2009; Gaines et al., 2010). Glyphosate resistance in transgenic plants can also result from glyphosate inactivation by oxidation (*gox* gene) (Dill, 2005). Some or all of these mechanisms may result in glyphosate resistance in GRL1, GRL65, and GRT20, since these mutations showed segregation as a single dominant gene (Table 2). For instance, shikimic acid accumulated in WTL, but not in GRL1, in a pattern indicative of amino acid changes in EPSPS (Fig. 2). No previous report has shown glyphosate resistance as a result of loss-of-function mutation, although a transporter loss of function may confer resistance due to decreased glyphosate translocation or absorption.

Naturally occurring glyphosate resistance in weeds can result from increased accumulation of the *EPSPS* transcript either as a result of regulatory mutations or as a result of increased copy number through gene amplification. Glyphosate resistance due to *EPSPS* gene amplification can segregate as a multiple-gene trait if there are copies on multiple chromosomes (Gaines et al., 2011). The GRL1 mutation clearly does not result from this mechanism because it neither shows increased expression of *EPSPS* nor shows segregation as a multiple-gene trait. Because GRL33 and GRH9 segregated as either semidominant or multiple-gene traits (Table 2), future work will need to examine whether these mutations are associated with elevated *EPSPS* mRNA levels. However, it seems unlikely that GRL33 and GRH9 result from *EPSPS* gene amplification, because EMS typically induces either point mutations or small deletions, not gene duplications (Shukla and Auerbach, 1981). Cloning and expressing the mutated EPSPS to explore the effects of the mutation on enzyme kinetics is also the subject of future work.

The GRL1 line contains a unique EMS-induced C715T point mutation resulting in a predicted amino acid substitution of L239F in *TaEPSPS-7D1*. The EPSPS mutations resulting in target-site resistance have been previously identified in the conserved P106 residue (or P172 of TaEPSPS). No one has previously identified a mutation affecting L239 that resulted in increased glyphosate resistance. The fact that GRL1 is a dominant trait and does not result in a high level of increased glyphosate resistance is consistent with the notion that the GRL1 phenotype may result from this point mutation in *TaEPSPS-7D1*. Shikimic acid accumulated in GRL1, consistent with an amino acid mutation. No mutations were observed in the sequenced portion of the *EPSPS* coding regions of GRL33 and GRH9. Since both of the mutants appear to be semidominant, they may result from a gain-of-function mutation. A gain-of-function mutation is most likely to occur within the enzyme (not signaling peptide) region of the protein. Thus, it is possible that resistance phenotypes of GRL33 and GRH9 mutants result from mutations in genes other than *EPSPS*.

Putative Protein Structure of the Mutated *TaEPSPS-7D1*

The amino acid substitutions at P106 of EPSPS change the enzyme structure within the active site, resulting in a reduced affinity for glyphosate to G101, the critical amino acid residue for glyphosate binding within the pocket of EPSPS (Zhou et al., 2006; Funke et al., 2009). It has been suggested that the large side chain of L106 can affect the conformation within the active site of enzyme, reducing glyphosate binding to G101. If the L239F amino acid change causes glyphosate resistance, then we might expect this amino acid residue to localize to the EPSPS active site.

We examined if the TaEPSPS-7D1-L239F allele may also cause a conformational change to the enzyme using computer modeling based on the crystal structure of *E. coli* EPSPS in complex with shikimate and glyphosate (using Swiss PDB Viewer; Guex and Peitsch, 1997). The mutated TaEPSPS-7D1-L239F protein was predicted to assemble an EPSPS structure similar to that of *E. coli*. The F239 residue corresponding to V164 of EPSPS in *E. coli* is located outside of the enzyme active site (marked with a red arrow in Supplemental Fig. S5). No previous evidence has suggested that the L239 (V164 in *E. coli*) residue of EPSPS may be involved in binding or interaction with glyphosate, phosphoenolpyruvate (PEP), or shikimate-3-phosphate (S3P) during enzyme catalysis (Padgette et al., 1991; Schonbrunn et al., 2001; Funke et al., 2009). However, two studies indicated that mutations in residues outside an enzyme active site could affect substrate-enzyme binding affinity due to altered enzyme binding pocket geometry and altered amino acid interactions during in catalysis, without changing the apparent three-dimensional folding structure of the enzyme (Jeffery et al., 2000; Mendonça and Marana, 2011). Future work will need to examine the effect of the L239F mutation on EPSPS enzyme kinetics (Schonbrunn et al., 2001).

Development of glyphosate resistance in wheat would augment and complement the currently available systems, particularly since currently available management systems built around herbicide resistance in wheat limit rotational flexibility. The use of glyphosate in other crops has led to the selection for glyphosate resistance in weeds, and careful consideration would need to be given to the merits of deploying glyphosate resistance in wheat—an obsolete technology in areas where transgenic

GR crops are already widely deployed. As previously mentioned, the most significant weeds competing with wheat are grass species common in areas where wheat is the only crop produced, such as wild oat, feral rye, downy brome, and jointed goatgrass. The use of glyphosate in wheat for control of such weeds may be possible if carefully stewarded.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

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