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Development of non-transgenic glyphosate tolerant wheat by TILLING

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Abstract

Glyphosate (N-phosphonomethyl-glycine) is the world's most widely used broad spectrum, post-emergence herbicide. It inhibits the chloroplast-targeted enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), a component of the plant and microorganism-specific shikimate pathway and a key catalyst in the production of aromatic amino acids. Variants of EPSPS that are not inhibited by glyphosate due to particular amino acid alterations in the active site of the enzyme are known. Some of these variants have been identified in weed species that have developed resistance to glyphosate because of the strong selective pressure of continuous, heavy glyphosate use. We have used TILLING (Targeting Induced Local Lesions in Genomes), a non-transgenic, target-selected, reverse genetics, mutation breeding technique, and conventional genetic crosses, to identify and combine, through two rounds of mutagenesis, wheat lines having both $T_{102}I$ and $P_{106}S$ (so-called TIPS enzyme) mutations in both the A and the D sub-genome homoeologous copies of the wheat EPSPS gene. The combined effects of the $T_{102}I$ and $P_{106}S$ mutations are known from previous work in multiple species to minimize the binding of the herbicide while maintaining the affinity of the catalytic site for its native substrates. These novel wheat lines exhibit substantial tolerance to commercially relevant levels of glyphosate.

Introduction

Glyphosate (N-phosphonomethy-glycine) is the world's most widely used herbicide [1, 2]. It forms the basis of the commercial Roundup® ready system in which multiple crops such as maize (*Zea mays* L.), soybeans [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.), canola (*Brassica napa* L.), alfalfa (*Medicago sativa* L.) and sugar beets (*Beta vulgaris* L.) have been rendered resistant to the effects of glyphosate-containing herbicides by genetic transformation with herbicide insensitive forms of the glyphosate target enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [3]. This enzyme is the penultimate enzyme of the shikimate pathway, which in plants, bacteria and fungi is responsible for the synthesis of aromatic amino acids and downstream metabolites [4]. In plants, glyphosate is rapidly translocated to the meristems and kills these growing points by starving them of these essential amino acids [5].

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: Authors C.P.M, M. N.S., J.C.M. and A.J.S. are co-inventors on US patent number 10,801,036 related to the described research entitled: "Wheat having resistance to glyphosate due to alterations in 5-enol-pyruvylshikimate-3 phosphate synthase" and all authors were employed by Arcadia Biosciences, which has a commercial interest in the described research. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Due to the strong selection pressure of continuous, heavy use of this herbicide in glyphosate-resistant crops, many weeds have developed resistance to glyphosate and multiple resistance mechanisms are known [6]. These include target site mutations in the EPSPS enzyme itself, as well as other mechanisms including gene amplification of EPSPS [7, 8] and mutations that increase vacuolar sequestration of glyphosate [9]. In some cases, weed species exhibit combinations of multiple resistance mechanisms [10, 11].

Among the target site mutations identified to date, missense mutations at amino acid 106-the numbering is based on the mature form of the *Arabidopsis* EPSPS-are most frequently found. The normally occurring proline at this position is altered to serine, threonine, valine and leucine in various glyphosate tolerant weed species (reviewed in: [12]). These single missense mutations impart a 2–6 fold increase in glyphosate tolerance. Greater glyphosate tolerance has evolved in goosegrass (*Eleusine indica*) with the combination of the $P_{106}S$ missense alteration with a $T_{102}I$ mutation (known as the TIPS enzyme) [13], which leads to an EPSPS enzyme that is essentially insensitive to the effects of glyphosate [14]. However, the TIPS enzyme in goosegrass is associated with a fitness cost due to its lowered catalytic efficiency [15, 16]. Other double mutant and even a triple mutant EPSPS have evolved in response to herbicide pressure [17, 18].

Glyphosate resistant wheat was generated transgenically [19], but, due to opposition by growers and consumers, was never commercialized or widely grown [20]. Nevertheless, a glyphosate-resistant wheat would offer benefits to growers in some geographies for post-emergent control of otherwise difficult to control weeds, including wild oats (*Avena fatua* L.), feral rye (*Secale cereal* L.), jointed goat grass (*Aegilops cylindrica*), downy brome (*Bromus tectorum* L.) and blackgrass (*Alopecurus myosuroides*). These grass weeds can be difficult to control with currently available options and impose considerable economic costs. For example, in the United Kingdom, it was recently estimated that blackgrass control, and the yield losses in winter wheat that it causes, may result in an annual economic burden as high as £1 billion [21]. These issues have prompted efforts to develop wheat resistant to glyphosate [22] and other herbicides [23–25] via non-transgenic (mutagenesis) methods that might be more acceptable to growers and the public. These efforts have had some success with the development of wheat lines with some glyphosate tolerance and wheat with tolerance to other herbicides through forward screening of mutagenized wheat seeds. However, the genetic basis of wheat glyphosate tolerance in the described glyphosate tolerant lines is incompletely understood [22] and the level of glyphosate tolerance is insufficient to be commercially useful.

Here, we describe our use of the non-transgenic method of TILLING [26–28] to create and identify non-transgenic target site mutations in the A, B and D sub-genome homoeologous copies of the wheat EPSPS gene. Through two rounds of mutagenesis, we were able to identify wheat lines containing both active site $T_{102}I$ and $P_{106}S$ mutations in the A and, separately, in the D genome copy of EPSPS (in wheat these amino acid positions are equivalent to $T_{168}I$ and $P_{172}S$ using the full-length wheat 7A EPSPS protein as reference (KP411547), [29], but for simplicity's sake we will use the conventional numbering based on the mature *Arabidopsis* or maize EPSPS sequence (CAA44974.1), [30]). We created lines containing both A and D sub-genome homozygous copies of this double mutant combination through the use of conventional genetic crosses and these lines exhibited substantial tolerance to commercially relevant levels of glyphosate in the greenhouse and in the field.

Materials and methods

Plant material

Hexaploid wheat (cv. Express) with mutations in the EPSPS gene in each of the 7A, 7D and 4A homoeologs were crossed to each other and to the parental cultivar. Different genetic classes

were identified by KASP genotyping (https://www.biosearchtech.com/support/education/kasp-genotyping-reagents) using probes developed to specific SNPs and genomic DNA isolated from seedling leaf tissue. Wheat plants were sown in Sunshine Mix #3 and grown in pots in Conviron chambers and in the greenhouse as described by Slade et al. [31].

Preparation of TILLING library

Hexaploid wheat (*Triticum aestivum*), cultivar Express (Westbred) was mutagenized with EMS as follows: seed batches of 100g -500g (~2000–10000 seeds) were vacuum infiltrated in deionized H_2O (*ca*. 1000 seeds/100ml of H_2O) for about 4 min. Two of the small batches (100g) were then treated with EMS (Sigma) at a concentration of 0.75% (v/v) while all the other batches were treated with EMS at a concentration of 1% (v/v) for 18 hours. Following the EMS treatment, the seeds were washed for 4–8 hours in tap water. Subsequently an estimated 50,000 treated seeds were planted in a field (Vacaville CA, from November to December 2001) along with untreated control seeds. These M1 plants grew over the winter-early spring and circa 12,000 plants were harvested individually (May 2002). M2 seeds were catalogued. M2 seeds were then germinated in a greenhouse for leaf tissue collection and DNA preparation. A total of *ca*. 8,000–10,000 plants produced M3 seeds.

Selections for re-mutagenesis

Homozygous plants carrying EPSPS mutations $P_{106}S$ and $T_{102}I$ in genomes A and D, respectively, were selected and propagated to increase progeny seeds. The resulting seeds were then re-mutagenized with EMS as described above except that the exposure time to the mutagen was reduced from 18 hours to 15 hours. The seed germination rates varied from 20%-40% depending on the treated seed batch. The re-mutagenized plants were grown under greenhouse conditions. Leaf tissue samples were collected and used for DNA extraction. Progeny seed were harvested and catalogued as previously described.

TILLING and PCR

DNA collection from the TILLING population and PCR conditions have been previously described by Moehs et al., 2019 [32]. In addition, TILLING of wheat was conducted according to Slade et al., [28]. To summarize, all of the individual M2 mutant genomic DNAs (about 10,000) were screened in pools of two individual plants. The DNA concentration for each individual within the pool was approximately 2 ng/μl with a final concentration of 4 ng/μl for the entire pool. Then, 5 μls of the pooled DNA samples (or 20 ng wheat DNA) were arrayed on microtiter plates and subjected to gene-specific PCR. Amplification and TILLING was performed exactly as described [32]. For EPSPS expression analysis, RNA was extracted from leaf segments using the Qiagen RNeasy kit and cDNA was synthesized with the Superscipt kit from Invitrogen. (Carlsbad, CA). These methods are described in detail in Slade et al., [31].

Assessment of glyphosate resistance

For growth chamber tests, wheat seeds (3–10 seeds, replicated at least three times) were germinated in glass tubes with Phytagel medium containing or lacking 0.15mM glyphosate (Sigma-Aldrich). Plantlets were grown for two weeks in Conviron growth chambers under 18 hour illumination. For greenhouse spray chamber experiments (Fig 5), three week old seedlings mostly at the 5-leaf stage, grown in Sunshine Mix #3, were sprayed with a Teejet 9502EVS Nozzle at a 95 degree spray angle at 0.2 GPM (based on 40 psi), 21 inch above the edge of the flat. Roundup PowerMAX® with 47.8% active ingredient was diluted to the appropriate

equivalent gram acid equivalent/hectare concentration. Plants were sprayed with concentrations of glyphosate ranging from 432–1102 grams acid equivalent/hectare. This is equivalent to 11–28 fluid ounces/acre. Images were taken at one week intervals post spray. For the statistical analysis of results, ANOVA implemented in Graphpad Prism software using a Tukey's comparison was conducted. An alpha of $< = 0.05$ was considered significant.

Results

Identification of novel EPSPS TILLING alleles and glyphosate resistant phenotype

At the commencement of this project, the wheat genome sequence was not available and only cDNA and EST sequences putatively encoding wheat EPSPS homoeologs were available in public databases. We queried available wheat nucleotide resources, including genomic and EST databases at NCBI, with the known rice EPSPS genomic and cDNA sequences [33] to identify homologous sequences in wheat. The ESTs were downloaded from NCBI and assembled using DNASTAR Lasergene software. On the assumption that the intron-exon structure of the rice EPSPS gene and the wheat EPSPS gene were likely to be similar, we designed primers near the likely N-terminal region of wheat EPSPS exon 2 and sequences expected to be near the C-termini of wheat EPSPS exons 4 and 5 (Fig 1). Since the known conserved active site region of the wheat EPSPS gene, like the rice EPSPS gene, was likely to be present near the C-terminal end of the 2nd exon, the primer pairs we designed were intended to encompass this active site region. We used these primers to amplify PCR bands from genomic DNA of hexaploid bread wheat and these bands were cloned and sequenced. The sequenced bands fell into three sequence classes that putatively arose from an EPSPS gene on each of the three homoeologous genomes of hexaploid wheat. The sequences exhibited SNPs near the active site of the enzyme that clearly distinguished the different homoeologous copies of the gene (Fig 1). Isolation of RNA from leaf tissue and sequencing of cDNA with EPSPS-specific primers identified

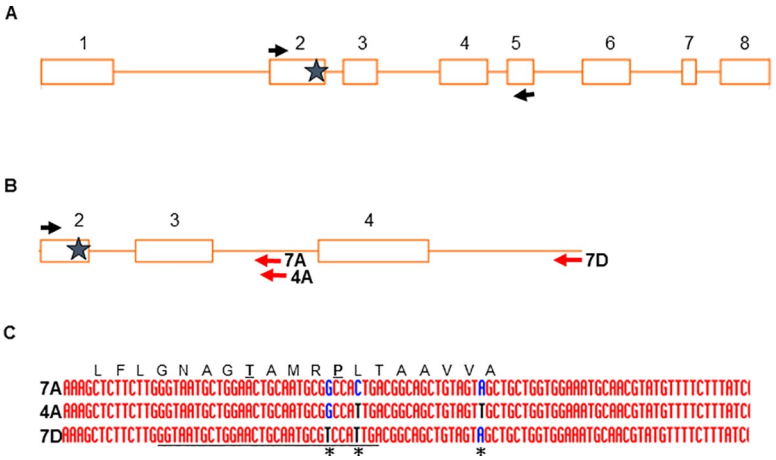

Fig 1. Overview of wheat EPSPS homoeolog cloning and TILLING strategy and homoeolog-specific SNPS. A. The genomic intron-exon structure of the rice EPSPS is shown. Boxes indicate the eight exons and lines represent introns. The black arrows indicate the locations of PCR primers used to amplify fragments of wheat EPSPS homoeologs from wheat genomic DNA spanning the active site of the enzyme-indicated with a star. B. TILLING of EPSPS homoeologs was accomplished with a common left primer (black arrow) and homoeolog-specific right primers (red arrows). C. Alignment of the active site regions of wheat EPSPS homoeologs showing homoeolog-distinguishing SNPs-indicated by asterisks. Key active site residues threonine and proline are underlined. Alignment created using default parameters of the software: Multalin (http://multalin.toulouse.inra.fr/multalin/).

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Table 1. Primer pairs used for gene amplification, TILLING and genotyping.

Common Homoeolog EPSPS Left primer	ACAGTGAGGATGTCCACTACATGCTTGA
Common Homoeolog EPSPS Right primer	AAATAGCTCGCACTTGAGGCATCACCTT
Chr 7A EPSPS Homoeolog Right primer	ACTTCTCTGACAGAGAACAGAAGTGTGCAC
Chr 4A EPSPS Homoeolog Right primer	TTGTGTAAGGTCGCATTGATCGTACTACCA
Chr 7D EPSPS Homoeolog Right primer	GAAAACTAGAATCATGCTTTTGTACTCCACTATC

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mRNA fragments homologous in sequence to each of the three homoeologs, indicating that each of these homoeologs is expressed. We did not find evidence for expression of more than three copies of EPSPS. Based on the sequences of the different SNP-containing EPSPS gene fragments, we designed new primer pairs that specifically amplified each of the individual homoeologous sequences (Table 1, Fig 1). These new homoeolog-specific primers exploited sequence differences, principally in the introns, that distinguished the homoeologs. Additionally, we confirmed that the different genomic sequences we had isolated arose from different homoeologs by amplifying these fragments in chromosome deletion lines of wheat. This determined that EPSPS sequence variant A is present on wheat chromosome 7A, variant B is encoded by chromosome 4A-due to a translocation [34], and variant D is present on homoeologous chromosome 7D (Fig 1). The chromosome assignments of these sequences have been determined by others as well [29]. Next, the identified primer pairs that specifically amplified single homoeologs were used to screen an existing TILLING resource of wheat cultivar Express [28] to identify novel SNPs in each of the wheat EPSPS homoeologs.

Fig 2 highlights the priority TILLING alleles identified in the active site of the enzyme in the first round of screening (underlined: $P_{106}S$ in the 7A genome and $T_{102}I$ in the 7D genome). These alleles represent just two of the more than 50 alleles identified across the fragments screened. Because of previously published information about missense mutations that render EPSPS resistant to the effects of glyphosate, we focused our search for alleles on amino acids threonine 102 and proline 106. The combination of two mutations at these sites, $T_{102}I$ and $P_{106}S$, leads to an EPSPS variant referred to as the TIPS enzyme that is insensitive to the effects

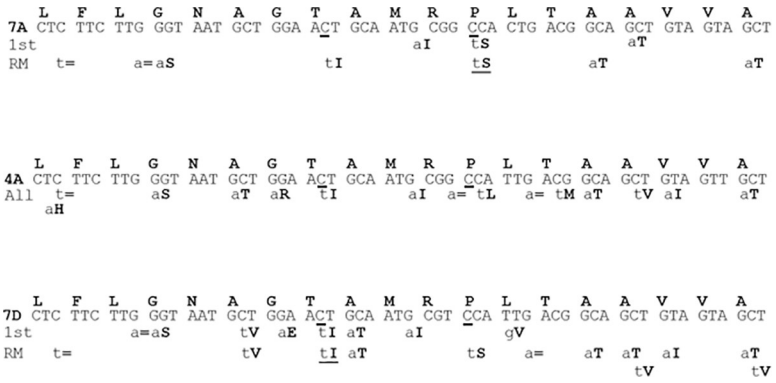

Fig 2. Depiction of TILLING alleles identified in the active site regions of wheat EPSPS homoeologs. Conserved regions around the active site of wheat 7A, 4A and 7D EPSPS homoeologs are depicted with identified novel induced SNPs shown below the homoeolog-specific DNA sequence. The 1st row refers to the TILLING screen of the initial mutagenized population. The second row labeled "RM" refers to alleles identified upon re-mutagenesis. The nucleotides mutated to create the "TIPS" alleles are underlined. Lower case letters indicate the altered nucleotide while the capitalized bolded letters refer to the amino acid alteration due to the SNP. Since the line that was re-mutagenized contained the 7A $P_{106}S$ and the 7D $T_{102}I$ alleles, these alleles are repeated in the "RM" row to indicate their presence in the re-mutagenized 2nd TILLING population. All of the TILLING mutants identified in the 4A EPSPS homoeolog in both TILLING screens are shown on one row. The equal sign indicates a silent SNP that doesn't alter the amino acid.

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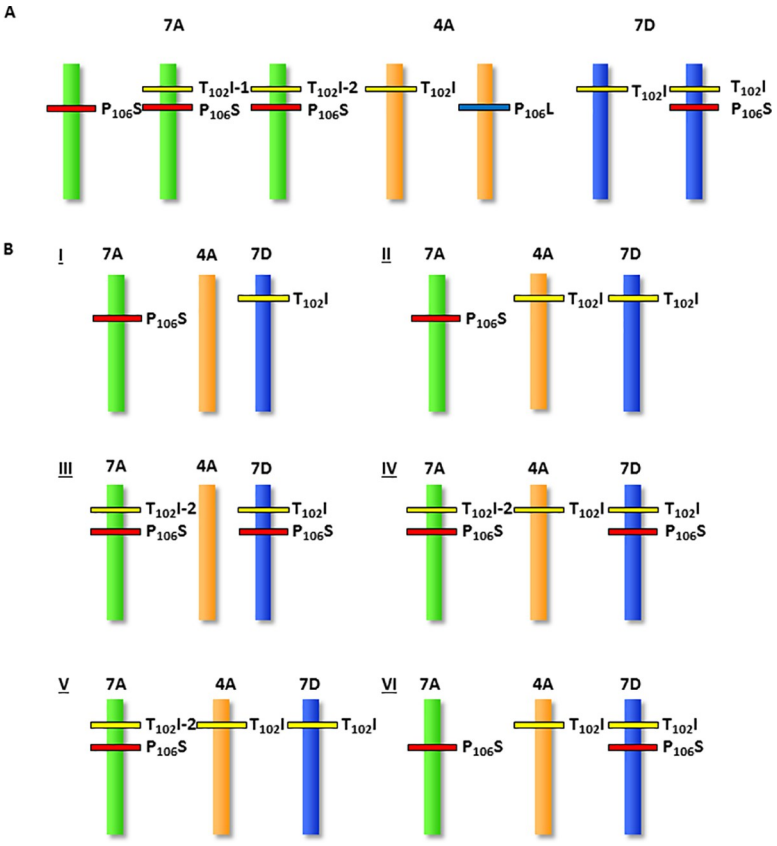

Fig 3. Schematic illustration of priority alleles identified and genetic constitution of lines created following conventional crosses. A. Priority alleles identified in the three targeted EPSPS homoeologs. The $T_{102}I$ allele on chromosome 7A was identified two independent times. B. Genetic constitution of wheat lines generated by conventional crosses. One chromosome is schematically illustrated but all lines are homozygous for the indicated alleles. (Not all possible genetic combinations are shown.).

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of glyphosate [13, 14, 35]. Each of these individual mutations also affects the enzymatic properties of EPSPS. Novel TILLING alleles that were not near the active site of the EPSPS enzyme were not characterized further. The $T_{102}I$ allele in the D genome was the first priority allele identified, followed by the $P_{106}S$ allele in the A genome. Subsequently, we also identified a $T_{102}I$ allele in the EPSPS homoeolog on chromosome 4A. In each case, the identified mutant was heterozygous, which allowed us to identify segregating sibling homozygous mutant and wild type EPSPS plants in the M3 generation using KASP (Kompetitive allele specific PCR; https://www.biosearchtech.com/support/education/kasp-genotyping-reagents) primers developed to distinguish them.

Homozygous lines containing each of the single 7A, 4A and 7D mutations as well as combinations containing two or three of the single mutations combined through conventional crosses were created (schematically illustrated in Fig 3) and initial glyphosate tolerance tests were conducted. Seeds homozygous for individual and combined priority alleles were germinated on glyphosate-containing medium and their ability to germinate and grow on this medium was determined (Fig 4). Subsequently, these lines were also grown in the field and sprayed with several levels of glyphosate-containing herbicide. The results indicated that the lines containing priority alleles could survive and continue to grow on medium, and set seed in the field, on levels of glyphosate and glyphosate-containing herbicide that were lethal to un-

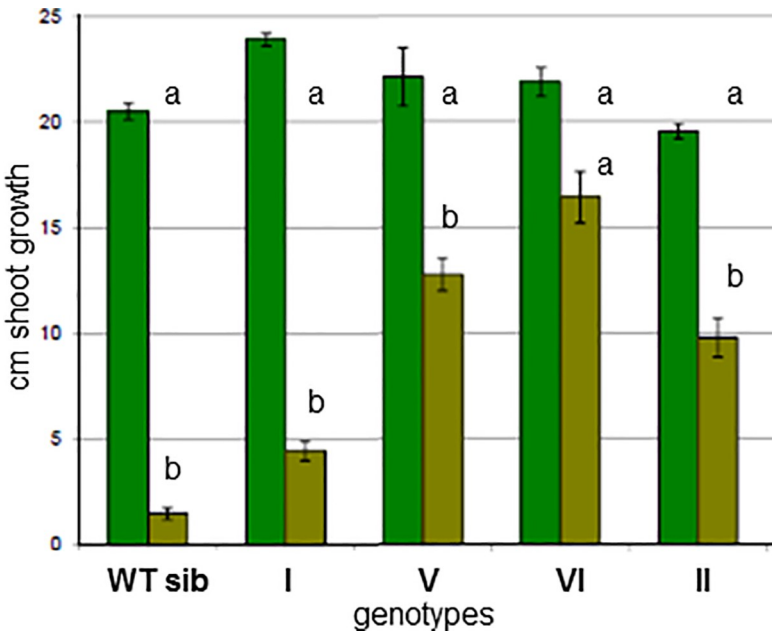

Fig 4. Shoot growth of different genotypes on Phytagel medium lacking or containing glyphosate. Average shoot height after twelve days is shown. Dark green bars represent medium without glyphosate. Light green bars indicate medium with 0.15mM glyphosate incorporated. Average +/- SE of six biological replicates is shown. Legend: WT sib: a wild type sibling line of a triple mutant line (genotype II in Fig 3B). I represents a double mutant line (genotype I in Fig 3B). V represents a line containing TIPS mutations on chromosome 7A and $T_{102}I$ mutations on both 4A and 7D chromosomes (genotype V in Fig 3B). VI has the TIPS mutation on chromosome 7D and $P_{106}S$ on the 7A chromosome as well as the $T_{102}I$ mutation on chromosome 4A (genotype VI in Fig 3B). II represents a genotype containing three single mutations (genotype II in Fig 3B). Different letters indicate significant differences between the genotypes and treatments.

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mutagenized control parental cultivar Express as well as to wild type sibling lines. Nevertheless, it was apparent that the level of glyphosate tolerance was insufficient to be commercially useful. This led us to seek additional mutants that would increase glyphosate tolerance further, by re-mutagenizing the homozygous line containing the 7A and 7D homoelogous mutant EPSPS alleles (Fig 3B, line I).

Re-mutagenesis, identification of additional EPSPS TILLING alleles and genetic crosses

The D genome $T_{102}I$ mutant EPSPS-containing line was crossed to the un-mutagenized parental variety, Express, and M2 mutant homozygotes were crossed to M3 homozygous progeny of the A genome $P_{106}S$ EPSPS mutant line. To generate a large amount of seed from A and D mutant EPSPS-containing homozygous progeny, M2 double homozygotes were identified, M3 seed was planted in the field and M4 seed was harvested. This M4 seed was used to generate a second (doubly mutagenized) TILLING population of approximately 10,000 individual M1 and an equivalent number of M2 members. Screening this population for additional alleles in the A and D genome copies of EPSPS led to the identification of new alleles: we identified an individual plant that in the background of the 7D genome $T_{102}I$ mutation also contained the nearby (11 nt away) $P_{106}S$ mutation. In addition, we identified two independent instances of plants containing the same TIPS mutant combination in the 7A genome copy of EPSPS. A plant containing a $P_{106}L$ mutation in the EPSPS homoeolog on chromosome 4A was also identified. An M4 plant homozygous for the 2nd independently identified TIPS EPSPS

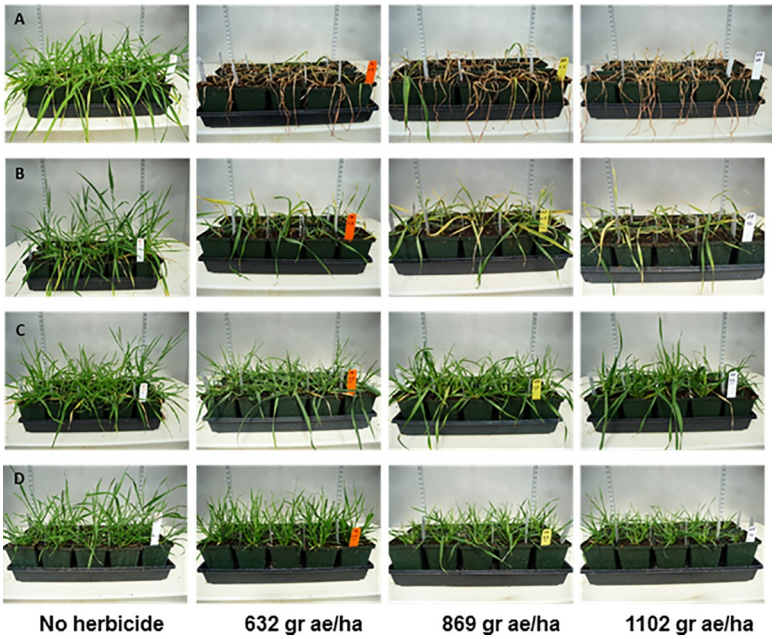

Fig 5. Greenhouse glyphosate spray chamber tests of different wheat genotypes. Row A represents the unmutagenized parental cultivar Express. Row B is the homozygous triple mutant line (genotype II in Fig 3B). Row C shows the genotype with the homozygous TIPS alleles on chromosomes 7A and 7D EPSPS homoeologs (genotype III in Fig 3B). Row D represents the genotype with TIPS mutations as well as the chromosome 4A T_{102}I allele (genotype IV in Fig 3B). Flats contained ~30 plants for each genotype except row B whose flats contained 13 plants. Flats were sprayed at the 5-leaf stage with the indicated rate of grams acid equivalent/hectare of Roundup PowerMAX®. Images were taken two weeks after herbicide application.

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chromosome 7A mutant combination was crossed to a plant homozygous for the three single mutants (7A_P_{106}S; 4A_T_{102}I; 7D_T_{102}I) in order to also introduce the chromosome 4A genome mutant. The TIPS chromosome 7D mutant combination was likewise crossed in the same manner as the TIPS 7A mutant to the above triple mutant. Finally, in order to create plants containing the TIPS mutants on both chromosome 7A and 7D EPSPS genes, as well as plants that also contained a chromosome 4A T_{102}I mutant, these two F1 plants, one heterozygous for the TIPS 7A mutants, and the other heterozygous for the TIPS 7D mutants were crossed to each other. F1 seeds from this cross were planted and genotyped and plants heterozygous for both 7A and 7D TIPS mutants were crossed to parental Express to reduce unwanted background mutations in these TILLING lines. This process of removing background mutations is ongoing and is currently at the BC3 generation. Crosses to remove background mutations were made between F1s rather than F2s to save a generation and speed this process. A schematic illustration depicting the principal priority alleles found and the genotypes of the lines that were created is shown in Fig 3.

Assessment of glyphosate tolerance of the identified alleles and allele combinations

All of the lines containing single 7A, single 7D or single 4A genome mutant alleles, as well as their wild type sibling lines were first screened *in vitro* on glyphosate-containing phytagel medium before being grown in the field along with their EPSPS wild type siblings and unmutagenized Express cultivar control. These experiments revealed that, individually and combined, the EPSPS mutant seeds-whether A genome P_{106}S or D genome T_{102}I-survived and

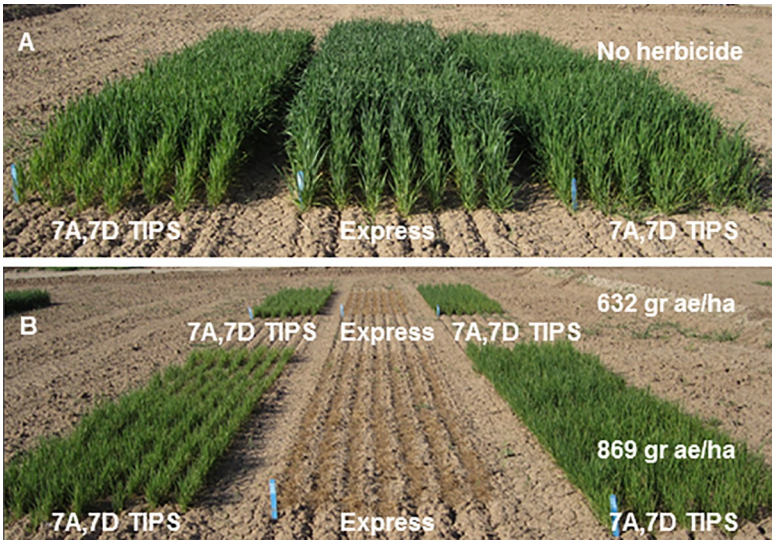

Fig 6. Field plots of wild type and TIPS allele containing lines without (A) and with (B) glyphosate herbicide treatment. In both panels A and B, the middle plot consists of the parental "Express" cultivar, while left and right plots are two separate lines containing chromosome 7A and 7D TIPS alleles (genotype III in Fig 3B). Panel A represents unsprayed plots, while in the foreground of panel B, plots were sprayed with the equivalent of 869 grams acid equivalent/hectare Roundup PowerMAX®. Plots in the background of panel B were sprayed with the equivalent of 632 grams acid equivalent/hectare Roundup PowerMAX®.

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grew when plated on low levels (0.15mM) of glyphosate-containing phytagel medium, including the growth of roots directly into the medium (not shown), while the wild type siblings did not survive (Fig 4). The double and triple single mutation lines (I and II in Fig 3B) were also grown in a field trial. This trial revealed that although these mutations enabled the plants to survive doses of glyphosate herbicide (632 grams acid equivalent/hectare Roundup PowerMAX®) that were lethal to un-mutagenized parental control cultivar Express and to wild type (at the EPSPS locus) siblings of the mutants, the seed yield of the mutants was depressed in comparison to the mutants unsprayed by the herbicide. In addition, the mutants exhibited a yield depression, in the absence of herbicide treatment, compared to the parental cultivar, presumably due to the general effects of the mutagenesis treatments. In our experience with a range of TILLING mutants in other genes, these mutagenesis treatment effects can be largely eliminated with several crosses to un-mutagenized material.

The results of the *in vitro* and field experiments to judge the glyphosate tolerance of the first single mutant EPSPS combinations determined that these mutants did not confer sufficient glyphosate tolerance to the plants to be commercially useful. As the doubly mutagenized TILLING resource became available and the 7A and 7D genome TIPS mutants were discovered, we repeated the previous phenotyping experiments on these new mutants and their combinations. *In vitro* experiments (Fig 4, V and VI) indicated that TIPS mutant-containing lines showed more robust survival and root extension into the medium than the previously tested single mutants. Spray chamber tests (Fig 5, rows C and D) and field growth (Fig 6) of the homozygous 7A and 7D double TIPS mutant lines revealed substantially enhanced herbicide tolerance compared to the single mutant lines. The double TIPS mutant was able to withstand the effects of an 869 grams acid equivalent/hectare Roundup PowerMAX® treatment, a treatment that led to complete mortality of the parental Express cultivar. Almost complete mortality of parental Express was observed at 432 grams acid equivalent/hectare, and at 632 gr acid equivalent/hectare no parental Express wheat survived. Although the TIPS lines subsequently set seed in

the field following glyphosate treatment, additional removal of unwanted background mutations will need to be undertaken before we can definitively assess the yield and agronomic utility of these lines. Therefore, it is premature to assess the ultimate level of glyphosate tolerance achieved until additional breeding has been conducted.

Discussion

Here we describe the creation, identification and development via TILLING of wheat lines that have substantially increased tolerance to the herbicidal effects of glyphosate compared to the parental cultivar. These lines contain both chromosome 7A and 7D TIPS EPSPS alleles, while having a wild type EPSPS allele on chromosome 4A. The TIPS alleles, 11 nucleotides apart in the active site of this enzyme, render EPSPS insensitive to the effects of glyphosate and, similarly, plants containing these alleles have substantially increased resistance to this herbicide. Although others have generated wheat with partial glyphosate resistance, these wheat lines were identified through forward screens and the genetic basis of the resistance is not completely determined [22].

A feature of TILLING is the ability to estimate the probability of identifying a desired mutation based on the induced SNP frequency in the population and the population size. At the commencement of this project, with a TILLING population size of approximately 10,000 and a mutation frequency of 1/24kb [28], we estimated an 80% probability of identifying at least one of the desired mutations. In the event, we exceeded expectations, and one of the priority alleles, T_{102}I on the 7A chromosome, was identified twice in independent wheat lines (Fig 3A).

An additional feature of mutagenic treatment with the DNA-alkylating chemical ethyl methanesulfonate (EMS), which was used in the creation of our wheat TILLING resource, is that approximately 95% of induced SNPs are G-C to A-T transitions [36]. Thus, one can predict the spectrum of expected missense alterations in codons. In our work, we identified only two induced SNPs from more than one hundred sequenced mutations that were not expected EMS-induced G-C to A-T transitions, namely L_{95}H in the 4A EPSPS homoeolog, and L_{107}V in the 7D EPSPS homoeolog (Fig 2). While the two priority alleles, T_{102}I and P_{106}S, are expected missense changes, an additional allele that confers glyphosate insensitivity in some EPSPS enzymes, namely the missense G_{101}A alteration [30, 37], is not expected from mutagenesis with EMS, and represents a limitation of this mutagen. The two mutations we did identify in this glycine (G_{101}R on the 4A EPSPS homoeolog and G_{101}E on the 7D homoeolog; Fig 2) are expected G-C to A-T EMS-induced transitions. The effects of these mutations on the EPSPS enzyme and on the plants containing them remain to be fully characterized.

This work is another illustration of the practical utility of TILLING and the remarkable ability of wheat to tolerate a high mutation load, which was also demonstrated by the hundreds of new alleles identified in the targeted genes in our earlier work [28, 31, 32]. TILLING in wheat has been applied by others for additional practical breeding efforts, such as generating lines resistant to the powdery mildew pathogen [38]. Aside from the particular result of glyphosate tolerance, our work also serves as an example of the ability to use TILLING to identify multiple induced SNPs nearby in the same gene after two rounds of mutagenesis (Fig 2). In addition to the TIPS alleles, we found other double mutants in EPSPS including one combination of mutants on the chromosome 7D homoeolog two amino acids apart (A_{100}V, T_{102}I, 6 nucleotides apart) and another double mutant combination, also on the 7D homoeolog, in adjacent amino acids (T_{102}I, A_{103}T) only 2 nucleotides apart!

While we have not conducted *in vitro* enzyme assays of the wheat EPSPS containing the TIPS mutations, the phenotypic effects on wheat plants containing these alleles in response to glyphosate treatment imply that in wheat, as in other tested plants [20], these mutations render

the enzyme insensitive to glyphosate. Nevertheless, evaluation of EPSPS enzymes with the TIPS allele in other species [13, 30] demonstrates that these TIPS enzymes, while having several thousand-fold elevated glyphosate inhibitory concentrations (K_i), and near normal K_ms for their natural substrate, phosphoenolpyruvate, also exhibited reductions in their V_{max}, to a level of about 12% of the normal enzyme. The wheat TIPS EPSPS enzymes also have a lower V_{max} than the native enzyme. Suggestive evidence that this is the case comes from the fact that all plants having homozygous TIPS variants on the 7A and 7D homoeologs as well as the homozygous 4A T_{102}I allele exhibited reduced vigor. This suggests, both that the three homoeologous EPSPS genes are the only active gene copies encoding this enzymatic activity in wheat, and that wheat plants containing only mutant copies of this enzyme are impaired in their ability to supply the required products of the shikimate pathway. Additional experiments will be required to confirm this supposition. This result implies that one unmutated copy of EPSPS is required to minimize any potential fitness cost of these alleles and to maintain plant vigor in the absence of glyphosate treatment. Other possible combinations of novel EPSPS alleles including, for example, a wheat line with one TIPS and one wild type allele on either the 7A or 7D chromosome in combination with the chromosome 4A P_{106}L mutation (Fig 3) have yet to be characterized in detail.

The TILLING wheat lines we generated are not as resistant to glyphosate as transgenic glyphosate tolerant wheat [19] since the transgenic wheat was developed using a strong constitutive promoter to express the glyphosate-insensitive EPSPS gene. Nevertheless, our non-transgenic glyphosate-tolerant wheat is resistant to commercial levels of glyphosate herbicide. Non-transgenic glyphosate tolerant wheat may have uses in weed and disease control [39, 40] and this wheat may benefit from advancements in robotic weeding technology that enables targeted herbicide spraying [41]. In addition, the glyphosate tolerance may, in the future, be further improved by adding additional alleles in genes that generate glyphosate tolerance by independent mechanisms-e.g. reduced glyphosate translocation.

Although the advent of CRISPR/Cas9 and related gene editing methods to modify plant genomes has taken plant research by storm [42], and may supplant TILLING for some applications, the recent decision by the European Union to subject CRISPR-derived crops to regulatory requirements similar to transgenic crops [43] means that crops modified by TILLING have an easier path to market than CRISPR-derived crops in certain geographies. Finally, by expanding the conception of what can be achieved by conventional breeding, examples such as ours of identifying multiple missense mutations in the same gene may influence the discussion of the regulation of alleles derived by editing methods.

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