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TITLE:

Development of Targeting Induced Local Lesions IN Genomes (TILLING) Populations in Small Grain Crops by Ethyl Methanesulfonate Mutagenesis

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KEYWORDS

Mutagenesis, TILLING population, EMS, Reverse Genetics, Small Grains, Cel-1 endonuclease

SUMMARY:

Described is a protocol for developing a Targeting Induced Local Lesions IN Genomes (TILLING) population in small grain crops with use of ethyl methanesulfonate (EMS) as a mutagen. Also provided is a protocol for mutation detection using the Cel-1 assay.

ABSTRACT:

Targeting Induced Local Lesions IN Genomes (TILLING) is a powerful reverse genetics tool that includes chemical mutagenesis and detection of sequence variation in target genes. TILLING is a highly valuable functional genomics tool for gene validation, especially in small grains in which transformation-based approaches hold serious limitations. Developing a robust, mutagenized population is key to determining the efficiency of a TILLING-based gene validation study. A TILLING population with a low overall mutation frequency indicates that an impractically large population must be screened to find desired mutations, whereas a high mutagen concentration leads to high mortality in the population, leading to an insufficient number of mutagenized individuals. Once an effective population is developed, there are multiple ways to detect mutations in a gene of interest, and the choice of platform depends upon the experimental scale and availability of resources. The Cel-1 assay and agarose gel-based approach for mutant identification is convenient, reproducible, and a less resource-intensive platform. It is advantageous in that it is simple, requiring no computational knowledge, and it is especially

suitable for validation of a small number of genes with basic lab equipment. In the present article, described are the methods for development of a good TILLING population, including preparation of the dosage curve, mutagenesis and maintenance of the mutant population, and screening of the mutant population using the PCR-based Cel-1 assay.

INTRODUCTION:

Point mutations in genomes can serve many useful purposes for researchers. Depending on their nature and location, these mutations can be used to assign functions to genes or even distinct domains of proteins of interest. On the other hand, as a source of novel genetic variation, useful mutations can be selected for desired traits using phenotyping screens and further used in crop improvement. TILLING is a powerful reverse genetics tool that includes chemical mutagenesis and detection of sequence variation in the target gene. First developed in *Arabidopsis*[1] and *Drosophilia melanogaster*[2], TILLING populations have been developed and utilized in many small grain crops such as hexaploid bread wheat (*Triticum aestivum*)[3], barley (*Hordeum vulgare*)[4], tetraploid durum wheat (*T. dicoccoides durum*)[5], diploid wheat (*T. monococcum*)[6] and the "D" genome progenitor of wheat *Aegilops tauschii*[7]. These resources have been used to validate the roles of genes in regulating abiotic and biotic stress tolerance[8], regulating flowering time[9], and developing nutritionally superior crop varieties[5].

TILLING, along with the use of alkylating mutagenic agents such as ethyl methanesulfonate (EMS), sodium azide, N-methyl-N-nitrosourea (MNU), and methyl methanesulfonate (MMS), has advantages over other reverse genetics tools for several reasons. First, mutagenesis can be conducted on practically any species or variety of plant[10] and is independent of the transformation bottleneck, which is particularly challenging in the case of small grains[11]. Second, in addition to generating knockout mutations that can be obtained by other gene validation approaches, a range of missense and splicing mutations can be induced, which can discern functions of individual domains of the proteins of interest[12]. Moreover, TILLING generates an immortal collection of mutations throughout the genome; thus, a single population can be used for functional validation of multiple genes. In contrast, other reverse genetics tools generate resources specific to only the gene under study[13]. Useful mutations identified through TILLING can be deployed for breeding purposes and are not subject to regulation, unlike gene editing, whose non-transgenic classification is still uncertain in many countries. This becomes especially relevant to small grains that are internationally traded[14].

TILLING is a simple and efficient gene validation strategy and requires mutagenized populations to be developed for investigating genes of interest. Developing an effective, mutagenized population is key to determining the efficiency of a TILLING-based gene validation study. A TILLING population with a low overall mutation frequency indicates that an impractically large population must be screened for desired mutations, whereas a high mutagen concentration leads to high mortality in the population and an insufficient number of mutagenized individuals. Once a good population is developed, there are multiple ways to detect mutations in the genes of interest, and the choice of platform depends on the experimental scale and availability of resources. Whole genome sequencing and exome sequencing has been used to characterize all mutations in TILLING populations in plants with small genomes[15,16]. Exome sequencing of two

TILLING populations has been performed in bread and durum wheat and is available to the public for identifying desirable mutations and ordering mutant lines of interest[17]. It is a great public resource in terms of availability of desirable mutations; however, in gene validation studies, the wild-type line should possess the candidate gene of interest. Unfortunately, it is still cost-prohibitive to sequence the exome of the entire TILLING population for reverse genetics-based validation of a few candidate genes in another background. Amplicon sequencing and Cel-1-based assays have been used in detecting mutations in targeted populations in wheat, and Cel-1 assays are simpler, requiring no computational knowledge, and are especially suitable for validation of a small number of genes with basic lab equipment[6,18].

In the present article, described are methods for the development of a good TILLING population, including preparation of the dosage curve, mutagenesis and maintenance of the mutant population, and screening of the mutant population using the PCR-based Cel-1 assay. This protocol has already been implemented successfully in developing and utilizing mutagenized populations of *Triticum aestivum*, *Triticum monoccocum*[6], barley, *Aegilops tauchii*[7], and several others. Included are explicit details of these methods along with useful tips that will help researchers develop TILLING populations, using EMS as a mutagen in any small grain plant of choice.

PROTOCOL:

1. Preparation of dosage curve for effective mutagenesis

1.1. Soak 100 seeds with the genotype of interest in six 250 mL glass flasks (100 in each flask) containing 50 mL of distilled water. Shake at 100 rpm for 8 h at room temperature (RT) for imbibition by the seeds.

1.2. In a fume hood, prepare 50 mL of 0.4%, 0.6%, 0.8%, 1.0%, and 1.2% (w/v) ethyl methanesulfonate (EMS) solution by dissolving 0.167, 0.249, 0.331, 0.415, and 0.498 mL of EMS in distilled water, respectively.

NOTE: EMS is liquid at RT with a density of 1.206 g/mL.

CAUTION: Use appropriate personal protective equipment (PPE) while handling EMS.

1.3. Decant the water out of five flasks and add 50 mL of EMS solution in each flask containing imbibed seed so that there are six different treatments with 0.0%, 0.4%, 0.6%, 0.8%, 1.0%, and 1.2% EMS solution. Shake flasks for 16 h at 75 rpm and RT.

1.4. Decant the EMS solution and collect the treated seeds separately for each treatment using cheese cloth. Inactivate the used EMS solution by adding one volume of EMS-inactivating solution (0.1 M NaOH, 20% w/v $Na_2S_2O_3$) for 24 h. Also treat the contaminated flasks and pipette tips with the EMS-inactivating solution for 24 h.

1.5. Wash the EMS-treated seeds under running tap water for 2 h. Transplant each seed individually into root trainers containing potting soil.

1.6. Grow plants at 20–25 °C under a 16 h light period.

1.7. Record data on plant survival after 15 days of transplantation. Use the following equation to calculate the survival rate for each treatment:

$$Survival\ rate = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ treated\ with\ EMS} \times 100$$

NOTE: If the germination rate is lower than 100% in controls, accurate survival rates in all the treatments should be calculated after subtracting the number of seeds that failed to germinate in the controls. A survival rate of 40%–60% is desirable for effective mutagenesis. It may be required to perform a second round of dosage optimization with a modified concentration according to the survival of the treated seeds until achieving the desirable lethality rate of 40%–60%.

2. Mutagenesis and maintenance of mutant population

2.1. Soak the final batch of at least 3,000 seeds dividing equally (600 seeds each) in five 1000 mL flasks containing 300 mL distilled water. Shake for 8 h at 100 rpm under RT for imbibition.

NOTE: The final size of the desirable population will depend on the mutation frequency and ploidy level of the genotype, but it is advisable to use at least 3,000 seeds for hexaploids, 4,000 seeds for tetraploids, and more than 7,000 seeds for diploids.

2.2. In a fume hood, prepare 1,500 mL of the optimized EMS concentration solution in distilled water.

2.3. Decant the water out of the flasks and add the optimal concentration of EMS solution in each flask containing 600 imbibed seeds. Shake the flasks for 16 h at 75 rpm and RT.

2.4. Decant the EMS and collect the treated seeds in cheese cloth. Inactivate the EMS solution and treatment containers with EMS-inactivating solution as done in step 1.4.

2.5. Wash the EMS-treated seeds under running tap water for 2 h. Transplant each EMS-treated M_1 seed individually into root trainers.

2.6. Grow M_1 plants (derived from M_0 seeds) at 20–25 °C under a 16 h light period.

NOTE: It may be required to vernalize the seedlings at the two leaf stage for 6 weeks at 4 °C, if the genotype of interest has a winter-type growth habit.

2.7. Allow the M_1 plants to self-pollinate, and harvest the M_2 seeds separately for each fertile M_1 plant.

NOTE: To avoid chances of potential outcrossing, cover the spikes of M_1 plants with pollination bags before anthesis.

2.8. Plant a single M_2 seed from each M_1 plant to avoid genetic redundancy.

2.9. Collect tissue from M2 plants at the two-leaf stage in 1.1 mL of racked 96 well microtubes. Collect around 80 mm of leaf tissue from each plant and record the ID of each sample in a tissue collection plan.

2.10. Freeze-dry the leaf tissue using a lyophilizer and store at -80 °C.

2.11. Maintain the M_2 plants at 20–25 °C under a 16 h light period.

2.12. Record data on mutant phenotypes of the M2 plants at regular intervals. The expected phenotypes are albino, chlorina, grassy shoot, variegated, partially fertile, sterile, etc.

2.13. Allow the M2 plants to self-fertilize and mature. Separately harvest and save the M3 seeds of the M2 plants (**Figure 1**).

NOTE: M3 seeds are used for validating the phenotype in reverse genetics studies. Therefore, M3 should be carefully catalogued and stored under cool and dry conditions. Alternatively, if the seed must be increased in field, head rows can be planted for each M3 plant, and M4 seeds from each row can be harvested and saved separately as the TILLING population resource.

3. Cel-1 assay for genetic characterization of mutants

3.1. Extract DNA from the leaf tissue of M_2 using a plant DNA extraction kit with a DNA purification system (see **Table of Materials**) following the manufacturer's recommendations.

3.2. Quantify DNA using a spectrophotometer and normalize the DNA concentrations to 25 ng/µL with nuclease-free water in 96 well blocks.

NOTE: It is important to check the quality of the DNA by running it on a gel, as low-quality DNA (smeared DNA) may result in false negatives in pooled samples.

3.3. Create 4x DNA pools by combining DNA from four 96 well blocks into one plate, while maintaining the row and column identity of each sample. Add 50 µL of DNA from each individual sample into the pool plate so that each pool plate well contains a total 200 µL of DNA from four different 96 well blocks.

3.4. Catalogue the identity of pooled DNA in the format of **Pool Plate-Row-Column** (e.g., Pool 1 A1 = Box1A1 + Box2A1 + Box3A1 + Box4A1).

3.4.1. Design genome-specific primers for the gene of interest using the Genome Specific Primers (GSP) page <https://probes.pw.usda.gov/GSP> with default settings for polyploid species. For diploids, use Primer 3 <http://primer3.ut.ee/> for designing primers using default settings. Design multiple primers, if needed, to cover the entire coding region of the gene of interest.

NOTE: The latest IWGSC assembly can be used to obtain sequences for the gene of interest in wheat using the URGI BLAST tool <https://wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST>. The optimal amplicon length is in the range of 800–1500 bp. **Table 1** shows examples of primers for waxy genes in hexaploid wheat.

3.5. Run a PCR for gene-specific primers on pooled DNA as follows:

3.5.1. Add 5 µL of PCR buffer, 2 µL (each) of 4 µM forward and reverse primers, 0.1 µL of DNA polymerase (see **Table of Materials**), 5 µL of pooled DNA template, then increase the volume to 25 µL using nuclease-free water.

3.5.2. Use a touch down profile to run the PCR reaction on a thermal cycler as follows: 95 °C for 1 min, seven cycles of 95 °C for 1 min, 67 °C to 60 °C for 1 min with temperature decreases of 1 °C per min and 72 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and final extension at 72 °C for 7 min.

NOTE: Above PCR profile works on wheat DNA template for most of the primers designed using primer 3 default settings. In case of non-specific amplification, PCR profile should be made stringent before moving to the next steps.

3.6. Generate heteroduplexes between mismatched DNA by incubating PCR products in thermal cycler using profile as follows: 95 °C for 2 min, five cycles of 95 °C for 1 s, 95 °C to 85 °C for 1 min with temperature decreases of 2 °C per cycle, and 60 cycles of 85 °C to 25 °C with decreases of 1 °C per cycle.

3.7. Add 2.5 µL of homemade Cel-1 endonuclease to the heteroduplexed PCR products and incubate for 45 min at 45 °C. Terminate the Cel-1 reaction by adding 2.5 µL of 0.5 M EDTA (pH 8.0).

NOTE: Cel-1 endonuclease can be extracted from fresh celery stalks using the protocol performed by Till et al.[19] It is very important to test the activity and optimum amount of Cel-1 endonuclease, which can be tested using previously characterized mutants or a commercially available mutation detection kit.

3.8. Run the Cel-1 treated products on a 3.0% agarose gel at 100 V for 2.5 h. The wells containing smaller and unique cleaved band(s), in addition to full-length uncleaved bands, will contain the mutant DNA sample.

3.9. Denconvolute mutant pools.

3.9.1. Follow steps 3.6, 3.7, 3.8, and 3.9 for individual M_2 DNA samples constituting the mutant pools identified in step 3.9.

3.9.2. To determine the zygosity of mutants, run two PCR (as described in step 3.6) for individual M_2 DNA, in which the first reaction contains 2.5 µL of M_2 DNA and 2.5 µL of wild-type DNA and the second reaction contains only 5 µL of M_2 DNA. If the mutation is heterozygous, an additional cleaved band will be present in both reactions. On the other hand, additional cleaved bands will be found only in the first reaction if the mutant is homozygous.

3.10. To identify the nature of the mutation, sequence the PCR products of the confirmed mutants using a Sanger sequencing platform following the manufacturer's instructions.

4. Calculation of mutation frequency

NOTE: Mutation frequency of a TILLING population refers to the average physical distance in which one mutation occurs in the individuals of that population. For example, a mutation frequency of 1/35 kb in a TILLING population means that an average individual of that population possesses 1 mutation per every 35 kb in the genome.

4.1. To determine the mutation frequency of a TILLING population, calculate the total number of bases screened.

4.2. To calculate the total number of bases screened, multiply the PCR product size by the total number of individuals screened.

4.3. Divide the total number of bases screened by the number of unique mutations observed using the following equation, which will yield the physical region possessing 1 mutation in the given TILLING population:

$$Mutation\ Frequency = \frac{(Product\ size - 100\ bp)x\ Number\ of\ individuals\ screened}{Total\ number\ of\ unique\ mutations\ observed}$$

NOTE: To account for the limitation in resolution of 50 bp at both the ends based on an agarose gel-based platform, subtract 100 bp from the product size in the calculation.

REPRESENTATIVE RESULTS:

Figure 2 shows the dosage curve of hexaploid bread wheat cultivar Jagger, diploid wheat *Triticum monococcum*[6], and a genome donor of wheat *Aegilops tauschii*[7]. The EMS doses for desired 50% survival rates were about 0.25%, 0.6% and 0.7% for *T. monococcum*, *Ae. tauschii*, and *T. aestivum*, respectively. The higher EMS tolerance of hexaploid wheat is due to its genome buffering capacity. However, despite both being diploid, EMS tolerance of *T. monococcum* was almost half that of *Ae. tauschii.* Therefore, these results underscore the importance of determining the appropriate EMS dose for individual genotypes of interest.

The presence of easily identifiable phenotypes in the M2 population confirms effectiveness of mutagenesis in small grain populations. The mutant phenotypes typically include albino, chlorina, stunted, grassy shoot, variegated, early/late flowering, partially fertile, and sterile. **Figure 3** shows some typical mutant phenotypes obtained in TILLING populations.

The Cel-1 assay and agarose gel-based approach for mutant identification is convenient, reproducible, and less resource-intensive platform. **Figure 4** shows a mutant identification using Cel-1 on agarose gel platforms. It should be noted that mutant DNA lanes contain unique patterns of cleaved band. The first round of 4x pool screening reduces labor needs, resources, and time expenditure. For instance, as shown in **Figure 4A**, four potential mutants were identified out of 48 individuals by performing PCR and the Cel-1 assay on 12 pooled samples. The deconvolution of mutant pools determined the zygosity of mutation and helped track the mutation down to individual samples (**Figure 4B**,**C**). **Figure 3B** shows the detection of heterozygous mutations in the A4 pool, as unique, cleaved bands are present in both Box 4-A4 and Box 4-A4+ wild-type DNA samples. On the other, the H5 pool contained homozygous mutations, as unique, cleaved bands are only present in the Box 5-H5+ wild-type DNA sample.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of developing EMS-mutagenized TILLING populations in small grain crops.

Figure 2: EMS dosage curve in three different species of wheat including *Triticum aestivum*, T. *monococcum*, and *Aegilops tauschii*.

Figure 3: Mutant phenotypes (yellow arrows) in various small grain M_2 TILLING populations. (**A**) An albino mutant in a barley M2 population, (**B**) chlorina mutant in a barley M2 population, (**C**) variegated mutant with pink discoloration in an *Ae. tauschii* M2 population, and (D) low tillering mutant in a *T. monococcum* M2 population.

Figure 4: Mutant identification in 4x pools following deconvolution using the Cel-1 assay and agarose gel-based approach. Shown is the (**A**) mutant pool in lane 7 with unique cleaved bands, (**B**) deconvolution of the heterozygous mutant pool, detecting the mutation in the A4 sample of DNA Box 4 with unique cleaved bands in Box 4-A4 and Box 4-A4+ wild-type DNA samples, and (**C**) deconvolution of the homozygous mutant, detecting the mutation in the H5 sample of DNA Box 5 with unique cleaved bands only in the Box 5-H5+ wild-type DNA sample.

Table 1: Primers for amplifying waxy genes in hexaploid wheat.

DISCUSSION:

TILLING is a highly valuable reverse genetics tool for gene validation, especially for small grains where transformation-based approaches have serious bottlenecks[11]. Developing a mutagenized population with a high mutation frequency is one of the critical steps in conducting functional genomics studies. The most important step in developing a robust TILLING population is to determine the optimal concentration of EMS. The 40%–60% survival rate in the M_1 has been found to be a good indicator of effectiveness of EMS mutagenesis in wheat and barley[4,6,18]. The surviving plants can provide decent mutation frequencies to help discover mutations in any gene of interest.

In rice, fertility of the M_1 plant is another determinant, in addition to survival of M_1 plants, and is reported to vary among different genotypes[20]. Hexaploid bread wheat and tetraploid durum wheat, on account of their polypoidy, have homoeoalleles in each genome for most of the genes, compensating for the loss-of-function of important genes due to mutations. This is known as genome buffering. Thus, polyploids can tolerate higher levels of EMS doses compared to diploids due to genome buffering[21,22]. However, it is known that tolerance of different diploid species to mutagens varies and may be regulated by diversity in genetic backgrounds. For example, *Ae. tauschii* showed a 55% survival rate at 0.6% EMS, whereas *T. monococcum* showed a 51% rate with 0.24% EMS; furthermore, any higher concentration in the latter species led to excessive plant death[6,7]. We have previously experienced that even in hexaploids, different cultivars tolerate different mutagen concentrations (data not shown). Furthermore, EMS tolerance varies significantly among different rice genotypes[20]. Therefore, it is highly recommended to obtain dosage curves for individual genotype of interest.

In order to analyze the efficacy of mutagenesis, several types of phenotypic mutants should be visible from the seeding stage to maturity of a TILLING population[7,23,24]. The phenotypic mutants to note include chlorina, albinos, variegated leaves, stunted, broad/narrow leaves, low/high tillering, early/late flowering, partially fertile, and sterile. Any deviation from the wild type phenotype represents a potential phenotypic mutant.

Since G and C are the primary target residues of EMS mutagenesis, there will be bias in the mutation frequencies of genes depending upon the GC content. A region with higher GC content will yield a high mutation frequency, whereas a region with low GC content will yield a low mutation frequency. To calculate the correct mutation frequency of a TILLING population, it is therefore suggested to obtain an average of two to three genes with varying GC content or normalize the rate of mutation to a 50% GC content[13].

The Cel-1 assay and agarose gel-based protocol described here are simple methods that do not require expensive instrumentation or complex analysis. However, it should be noted that this method is only suitable and efficient for mutation detection in a few genes. For screening mutations in a larger set of genes, multiplex amplicon sequencing method is recommended[3,24].

For a detailed protocol on multiple amplicon sequencing method, readers can refer to Tsai et al.[25] With advances in sequencing technology and reduced costs of sequencing, platforms such as exome capture have been used in characterizing mutations across a whole genome in the entire wheat TILLING population[17]. For plants with small genomes, even whole genomes of all individuals in the TILLING population can be sequenced[15,16]. However, the cost of screening all mutations in the individuals of a given TILLING population make it expensive perform whole-genome sequencing for a population developed for specific purposes. Therefore, for performing reverse genetics-based validation for a limited number of candidate genes in any laboratory with regular molecular biology instrumentation, Cel-1 based assays are a decent method of choice. Nonetheless, the choice of platform for detection of mutations is secondary to developing a TILLING population harboring multiple mutations throughout the genome. Therefore, the most critical step in the protocol is development of a robust TILLING population with a high mutation frequency.

DISCLOSURES:

Authors declare no competing financial interests.

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Figure 1

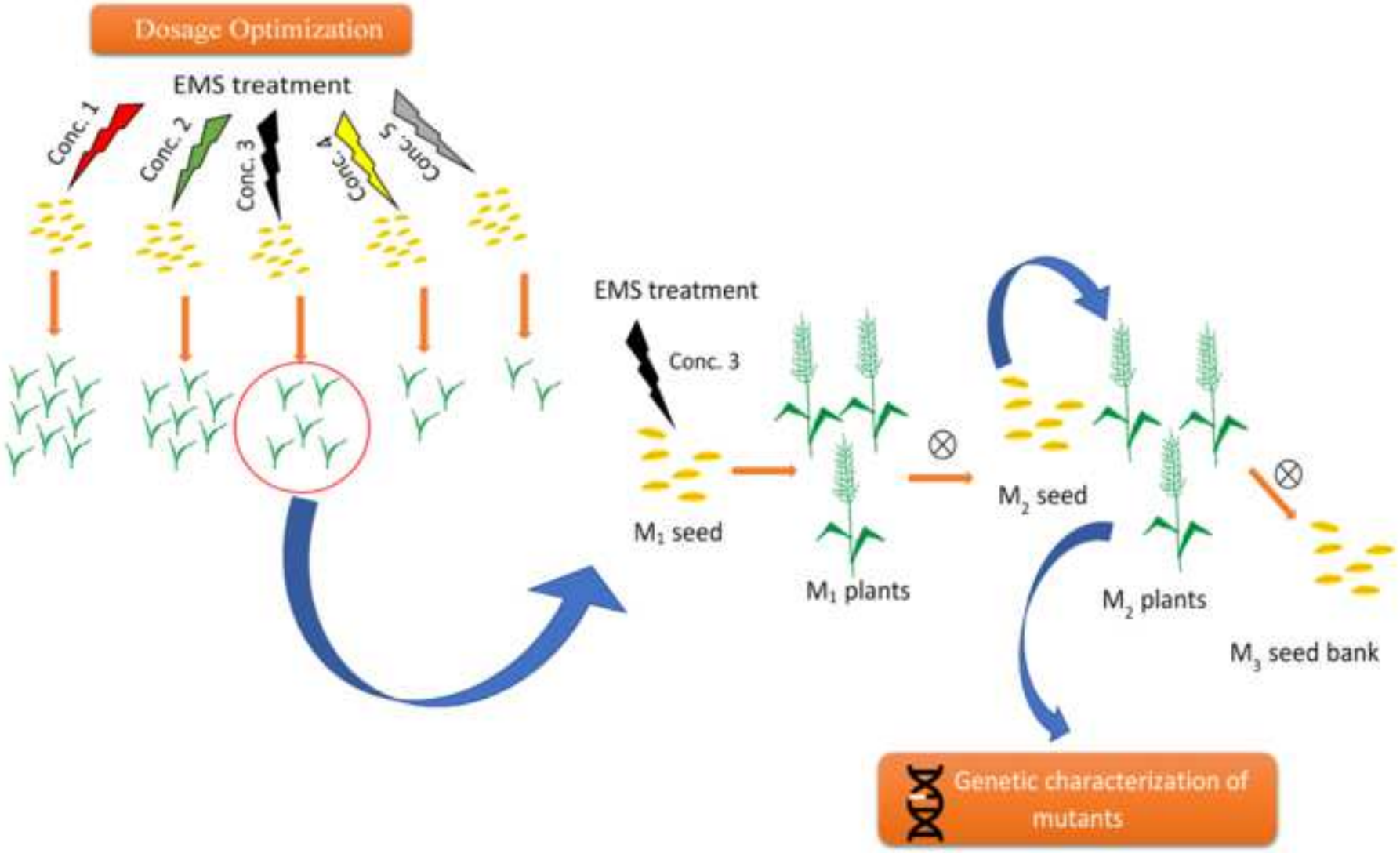


Figure 2

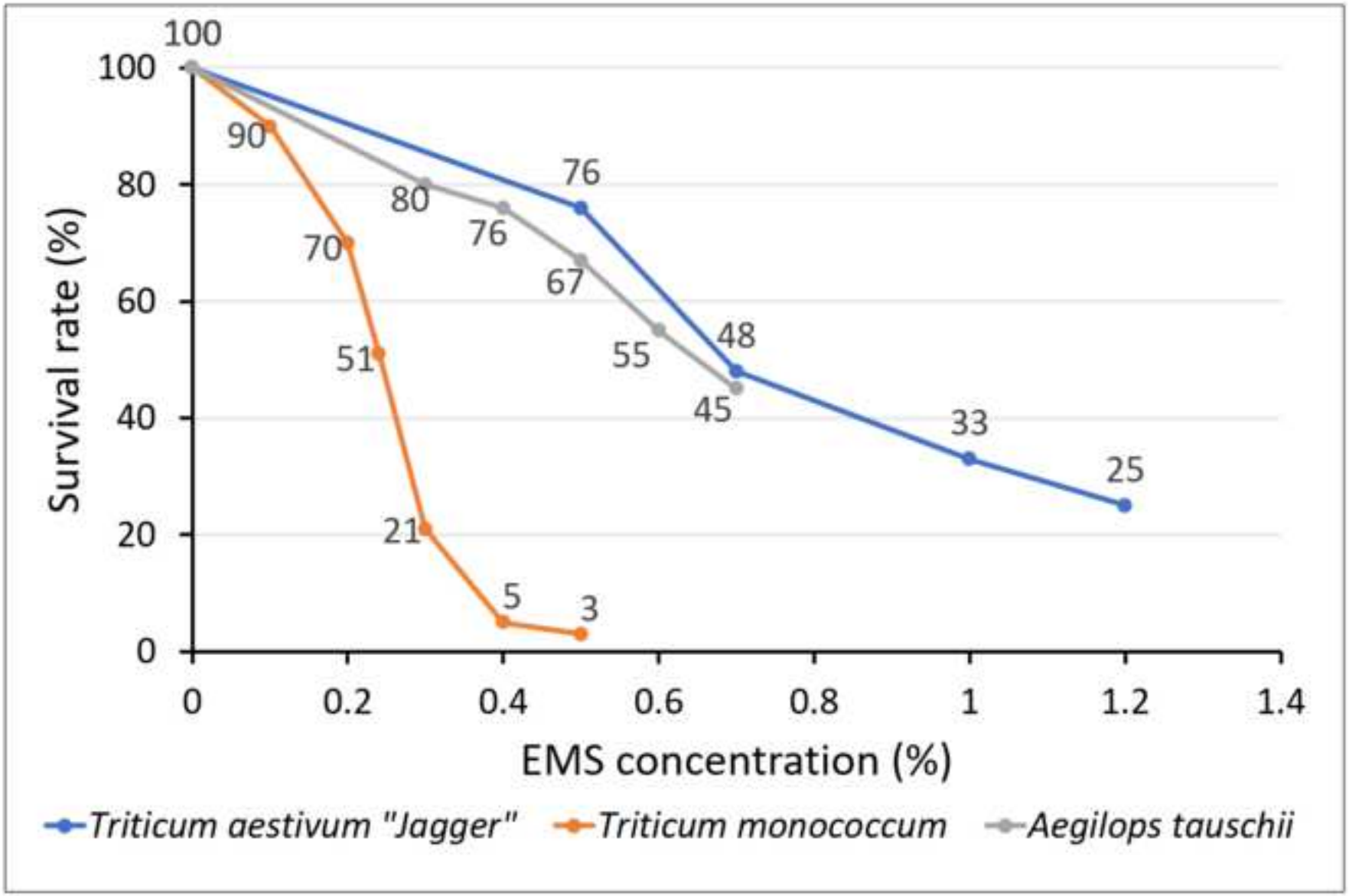


Figure 3

Click here to access/download;Figure;Fig 3.tif

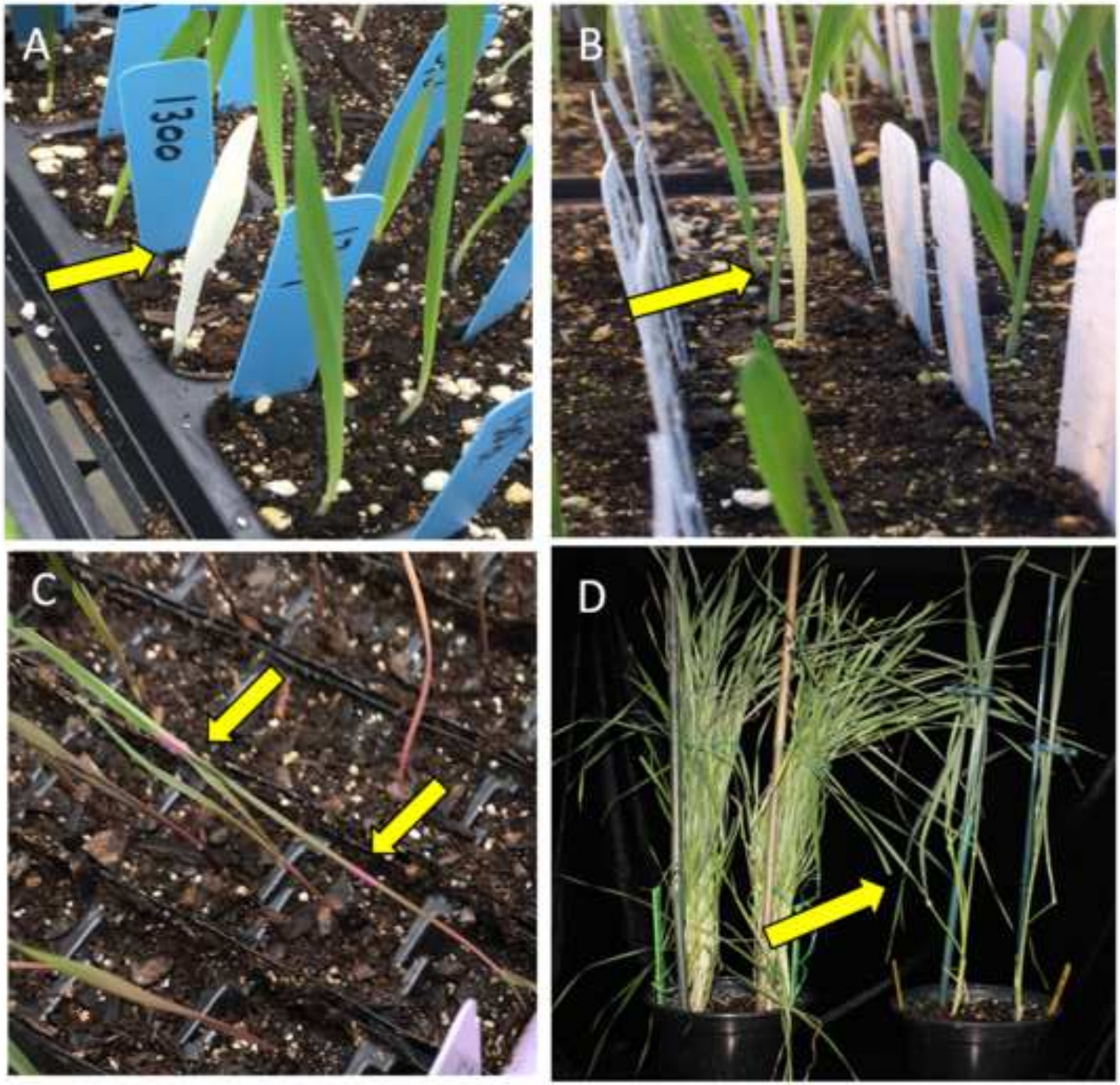
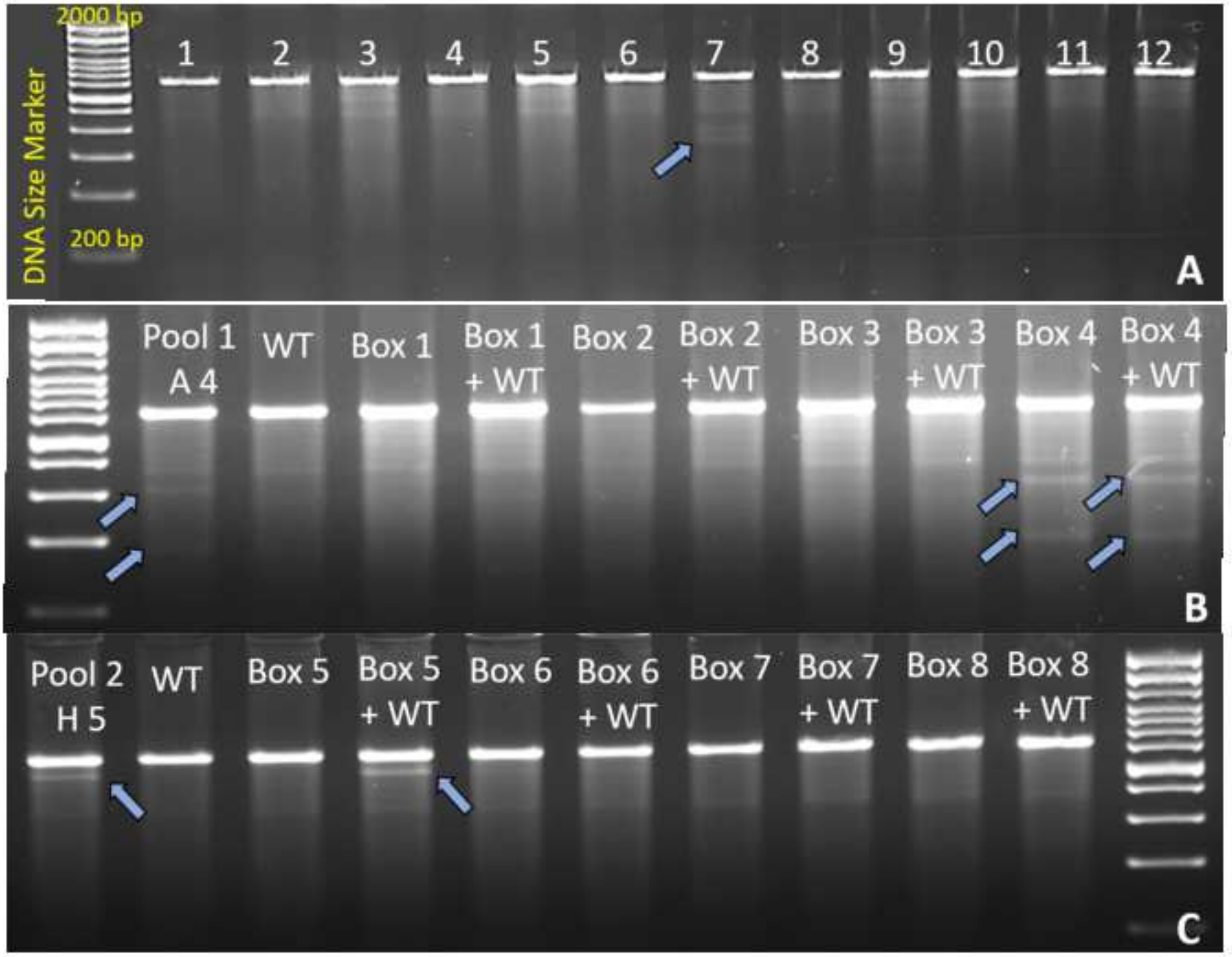


Figure 4

Click here to access/download;Figure;Fig 4.tif



Table

Gene	Primer name	Primer sequence (5'-3')	Product size
Waxy	Wx_AF	TCGCTCTGCATATCAATTTTGC	1022
	Wx_AR	GGAACTGGCAAGAAGGACTG	
Waxy	Wx_BF	GCGTCGTCTCCGAGGTACAC	870
	Wx_BR	GTCGAAGGACGACTTGAACC	
Waxy	Wx_DF	CCATGGCCGTAAGCTAGAC	1124
	Wx_DR	GTCGAAGGACGACTTGAACC	

Table of Materials

Click here to access/download;Table of Materials;Table TILLING_JoVE_Materials_sorted.xls

Name of Material/ Equipment	Company	Catalog Number
96 well 1.1 ml microtubes in microracks	National Scientific	TN0946-08R
Agarose I biotechnology grade	VWR	0710-500G
Biosprint 96 DNA Plant Kit	Qiagen	941558
Cel-1 endonuclease	Extracted as described by Till et al 2006	
Centrifuge 5430 R	Eppendorf	
Ethyl methanesulfonate	Sigma Aldrich	M-0880-25G
Freeze Dry/Shell freeze system	Labconco	
Kingfisher Flex purification system	Thermo fisher scientific	5400610
My Taq DNA Polymerase	Bioline	BIO-21107
Nuclease free water	Sigma aldrich	W4502-1L
NuGenius gel imaging system	Syngene	
Orbit Environ-shaker	Lab-line	
SPECTROstar Nano	BMG LABTECH	
T100 Thermal cycler	BIO-RAD	1861096

Comments/Description
For collecting leaf tissues
Kit for DNA extraction
Single strand specific endonuclease
EMS, Chemical mutagen
For lyophilization of leaf tissue
High throughput DNA extraction robot
Nano drop for DNA quantification

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Author(s): Lovepreet Singh, Adam Schoen, Alexander Mahlandt, Vijay Tiwari, Nidhi Rawat

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Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Answer: The manuscript has been thoroughly proofread for the spelling and grammar issues.

2. Please sort the Materials Table alphabetically by the name of the material.

Answer: The material table has been sorted alphabetically by the name of the material.

3. Figure 2: What are the yellow arrows signifying?

Answer: It has been noted in the figure legend now that the yellow arrows show mutant phenotype (L 336).

4. Figure 3: What are the ladders signifying? Native or denaturing gel? Etc.

Answer: The ladders are DNA size indicator ladders. The size of two bands of the ladder has been indicated in the figure. The gel is a regular 3% agarose w/v gel. It is not a PAGE, and is thus neither native nor denaturing.

5. Please use the degree symbol for temperatures instead of superscripted zeros and letter “o”s.

Answer: The superscripted zeros has been replaced with degree symbol.

6. Please do not abbreviate journal titles.

Answer: The full titles of journals has been included.

7. Please provide all DNA sequences in a separate Table.
Answer: The example of DNA sequences of waxy gene primers for hexaploid wheat has been included in Table 1.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript described the protocols for developing good TILLING populations (with a reasonable mutation frequency) in small grain crops by EMS muategensis including, the protocol used for the determination of a proper treatment dose, the treatment of seeds for the development of TILLING population, the procedure of mutant population development, and the detection of mutant pools and plants by Cel-I assay. The generalized protocols are applicable for most small grain crops, well-described and results are supported by previous reports. Here are a few concerns for clarification/improvement.

Major Concerns:

1. A "good population" is defined by the survival rate of seedlings in the present protocol, not by the "mutation frequency" as discussed to be critical in the manuscript (L92-94; L335). In practice, a ~40% lethal rate (relative to the control) would probably leads to high sterility of the plants, at least for some plant species, such as rice. Therefore, it would be useful to discuss this issue a bit regarding possible plant species differentiation.

Answer: The 40-60 % survival was not meant to define but indicate the possibility of generating good TILLING population by a quick assay for dosage optimization, which is not possible with M1 sterility parameter. Nonetheless, M1 sterility is an important indicator of the effectiveness of mutagenesis over all in the final bigger batch of the TILLING population. References towards this point have been included in the text now giving examples of rice mutagenized populations (L356-357, L368-369).

2. Similarly, based on published data of mutation frequencies in plant species with different ploidy levels, the authors recommended the amount of seeds to be treated (L159-161). Indeed, the fertility of M1 plants may also decrease with the decrease of ploidy level, in addition to the mutation frequency. This factor should be considered and discussed.

Answer: We have not used M1 sterility as a dosage determination parameter because: 1- It is not very practical to include a full generation worth of time to just select the optimum concentration of mutagen, and 2- On account of polyploidy, bread and durum wheat have genome buffering that preserves even deleterious mutations without significant penalty to the plants.

3. A few small grain crops are out-crossing or have a big rate of out-crossing, for such crops, attention needs to be taken in developing M2 populations using proper procedures to make M1 plants self-pollinated.

Answer: The procedure to avoid chances of outcrossing has been included as a note (L 187-188).

Minor Concerns:
1. Title: "small grains" might be better "small grain crops"

Answer: The title has been modified by substituting "small grains" with "small grain crops".

2. L124-128: Based on the information (the molecular weight of EMS and the amount used for preparation of EMS solution), it is not possible to reach the concentration of EMS, for example, by dissolving 0.24 mL EMS in 50 mL will lead to a concentration of (0.24 mL*1.206 g/mL)/50 mL = 0.579% (g/mL), not 0.4%.

Answer: We are grateful to the reviewer for identifying this error. The calculation error has been corrected (L124-126).

3. L148: It is not clear whether the "kill rate" is relative to ck or not, and how it is calculated (if it is a relative one).

Answer: The equation to calculate the survival rate has been included (L149-150). It has been mentioned in the note that how to use control in the calculations (L151-153).

4. L171 and elsewhere: M0 seeds are seeds before mutagenic treatment was performed, all seeds are referred as to M1 seeds after mutagenic (EMS) treatment.

Answer: The correction has been made (L177). The schematic of developing TILLING populations in small grain crops has been included as figure 1 to facilitate the understanding of the process.

5. L187: It would be better if details of lyophilization is given, or is it just keep the leaf tissue in a -80 degree Celsius freezer?

Answer: It is mentioned that the tissue is freeze dried using the lyophilizer (L196). The detailed protocol for lyophilization does not fall under the scope of this manuscript.

6. L189: It is not clear whether the seedling used for DNA extraction is grown further into plants (should be), a sentence should be added.

Answer: The sentence has been added (L198).

7. L221: add website for the Premier 3 program. Also the desirable length of amplicon should be indicated.

Answer: The website link for Primer 3 has been included (L232). The optimal amplicon length has been mentioned (L235).

8. L285/286: Removal of 50 bp seems only to be reasonable if the amplicons are longer than

1000 bp, for example; if the amplicons are shorter than 500 bp for example, it would be too much.

Answer: Since the desirable amplicon length is 800-1500 bp, above problem might not be encountered.

9. Citation of a few more relevant literatures of research on the rice plant, the very important staple food, small grain crop, is desirable.

Answer: Added as suggested.

Reviewer #2:
Manuscript Summary:
This protocol is quite useful for making the mutagenized populations in wheat and related species.

Major Concerns:
As the development of the wheat genome sequences, some information on how to get the sequences of the genes interested may be added in the PCR primer design.

Answer: The website of URGI BLAST tool with latest version of wheat genome sequence to obtain homologous sequences from wheat genome has been included (L233-235).

Minor Concerns:
Though the method using the CEL-I digestion and detection by Agarose gel is simple and applicable in small labs, but not very efficient, I suggest that some new methods, such as sequencing the amplicants of the mutants, may also be included.

Answer: The major focus of the article is to develop the TILLING population. Once a population with a high mutation frequency is developed, selection of platform for detection of mutations depends on the scale of the specific project. For example: skim sequencing, exome-capture, multiplex sequencing, Cel-1 based assays, High-Resolution melting curves are some of the options to consider depending upon the suitability of purpose. Moreover, since the format of JOVE is to present nuts and bolts of the protocols that have been tested and used by the researcher, therefore we have included Cel-1 based assays. We have explicitly mentioned the pros and cons of this approach. Readers have been directed to the source of the original detailed protocol on multiplex amplicon sequencing (L387-388) for TILLING by sequencing now.