A TILLING resource for functional genomics in *Arabidopsis thaliana* accession C24

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TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetic method that can be employed to generate allelic series of induced mutations in targeted genes for functional analyses. To date, TILLING resources in *Arabidopsis thaliana* are only available in accessions Columbia and Landsberg erecta. Here, we extended the *Arabidopsis* TILLING resources by developing a new population of ethyl methanesulfonate (EMS)-induced mutant lines in another commonly used *A. thaliana* accession C24. A permanent collection of 3,509 independent EMS mutagenized M2 lines was developed in *A. thaliana* accession C24, and designated C24TILL. Using the TILLING method to search C24TILL for mutations in four selected genes identified a total of 73 mutations, comprising 69.6% missense, 29.0% sense, and 1.4% nonsense mutations. Consistent with the propensity of EMS to induce guanine alkylation, 98.4% of the observed mutations were G/C to A/T transitions. Based on the mutations identified in the four target genes, the overall mutation density in the C24TILL collection was estimated to be 1/345 kb.

TILLING the *DUO POLLEN 1* (*DUO1*) gene from the C24TILL collection identified a truncation mutation leading to a deficiency in sperm cell differentiation. Taken together, a new TILLING resource, the C24TILL collection, was generated for *A. thaliana* accession C24. The C24TILL collection provides an allelic series of induced point mutations that will serve as a useful alternative reverse genetic resource for functional genetic studies in *A. thaliana*.

Key words: *A. thaliana* accession C24, DUO1, EMS, reverse genetics, TILLING

INTRODUCTION

Many reverse genetic resources have been developed for functional genetic studies. Because site-directed mutagenesis is not effective in plants, random mutagenesis approaches, including insertional (Wisman et al., 1998; Alonso et al., 2003), chemical (McCallum et al., 2000) and fast neutron mutagenesis (Li et al., 2001), have been used to establish reverse genetic platforms. In *Arabidopsis*, insertional mutagenic techniques using T-DNA or transposons have become popular tools for functional genomics. However, insertional mutagenesis often leads to complete gene knockouts, making it difficult to associate nuanced phenotypes with essential genes (Jander et al., 2002). Similarly, radiation mutagenesis, *e.g.*, fast neutrons, often induces large genomic deletions that affect multiple genes (Li et al., 2001). By contrast, classical chemical mutagenesis using a mutagen like ethyl methanesulfonate (EMS) induces an array of interesting point mutations with different impacts on gene function. Such allelic series are desirable because they generate a wide repertoire of mutant phenotypes covering a range of severity, which provide more insight into a gene's function. Moreover, individual plants carrying point mutations can be identified easily through a powerful method called TILLING (Targeting Induced Local Lesions IN Genomes).

TILLING is a reverse genetic method that takes advantages of classical mutagenesis, sequence databases, and high-throughput PCR-based screening for point mutations in a targeted sequence (Henikoff et al., 2004). The key advantage of TILLING over competing methods is that it can be applied to any plant species, regardless of ploidy level, genome size, or genetic background (Kurowska et al., 2011). TILLING extends genomic resources, particularly in organisms lacking reverse genetic tools, where mutants with a range of phenotypic severity are highly desirable. Since the inception of TILLING, this method has been applied to various organisms including *Cucumis melo* L. (González et al., 2011), *Solanum lycopersicum* (Minioa et al., 2010), *Brassica napus* (Wang et al., 2008; Harloff et al., 2012), *Brassica*
Additionally, mutations in the gene were evaluated from a functional standpoint. From each individual M1 plant, four M2 offspring were grown and a single fertile M2 plant was selected. In all, a population of 3,509 individual M2 plants was maintained. Genomic DNA and seed stocks were collected from these plants.

**DNA extraction and pooling** Genomic DNA was extracted from the leaves of M2 plants using a DNAeasy 96 Plant Kit (QIAGEN, USA) according to the manufacturer's protocol. Genomic DNA samples were quantified, diluted, and normalized to 1 ng/μl in 1/5 TE solution. Samples were stored at −80°C. Using a one-dimensional pooling strategy as previously described (Martín et al., 2009), diluted genomic DNA (1 ng/μl) was pooled 8-fold. Pooled genomic DNA was stored at −20°C for use in the subsequent TILLING screen.

**TILLING** Point mutations in targeted fragments were detected using the previously developed TILLING procedure (Till et al., 2006) with slight modifications. Each nested PCR reaction was performed in a 12.5 μl volume consisting of 1x PCR buffer (TaKaRa, Shiga, Japan), 0.2 mM dNTPs, 2 U ExTaq polymerase (TaKaRa, Shiga, Japan), 10 pmol forward and reverse unlabelled gene specific primers for the 1st PCR or 10 pmol forward (6-FAM labelled) and reverse (VIC labelled) common primers for the 2nd PCR, 5 μl of 0.5 ng/μl pooled genomic DNA for the 1st PCR or 1.0 μl of 1st PCR product for 2nd PCR and sterile 18 μl of M2 aliquot was added. The reaction was performed using a 96-well thermal cycler (Astech, Kyoto, Japan) programmed with the following conditions: 1) 94°C for 3 min; 2) 15 (1st PCR) or 25 cycles (2nd PCR) of 94°C for 30 sec, Tm-2°C for 30 sec, and 72°C for 90 sec; and 3) a final extension at 72°C for 7 min. The final PCR products were heated at 95°C for 10 min and slowly cooled (95°C ramping to 85°C at −2°C/sec, then 85°C ramping to 25°C at −0.1°C/sec) to generate heteroduplex PCR products. Five μl of the PCR reaction was treated with CEL1 SURVEYOR nuclease (Transgenomic, Omaha, NE) and incubated at 45°C for 15 min. Then, 5 μl of 150 mM EDTA was added. The CEL1-treated samples were cleaned through Sephadex G-50 resin (Amersham Pharmacia Biotech, Little Chalfont, UK) packed in 96-well Multiscreen-HV filter plates (Millipore, Billerica, MA). Next, the samples were mixed with 9.9 μl of 1% DAPI (4,6-diamidino-2-phenylindole dihydrochloride) and briefly mixed. The samples were heated at 95°C for 3 min before loading onto the ABI 3730xl (96-capillary) sequencer. Data were analysed using GENEMAPPER 4.0 fragment analysis software (ABI, Carlsbad, CA) and confirmed by DNA sequencing. The primer sequences are listed in Supplementary Table S1.

**Characterization of DUO1 mutant phenotype** Pollen grains from mature flowers were transferred into a microcentrifuge tube containing 200 μl DAPI staining solution (0.1 M sodium phosphate, pH 7.5; 1 mM EDTA; 0.1% Triton X-100; 0.4 μg/ml DAPI) and briefly mixed. Then, the stained pollen was transferred to a microscope
slide and observed under a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Silique length was measured and siliques were dissected using a 27.5-gauge needle (Becton Dickinson, Franklin Lakes, NJ) to observe seed setting.

**RESULTS AND DISCUSSION**

**Generation of the A. thaliana accession C24 mutant population** In order to develop a new reverse genetic resource, the commonly used A. thaliana accession C24 seeds were treated with EMS. Optimum EMS concentrations had been previously reported for A. thaliana Col-0 and Ler accessions as 20–45 and 20–50 mM, respectively (McCallum et al., 2000; Martín et al., 2009). Nonetheless, we began by assessing the proper EMS concentration for A. thaliana accession C24 seeds as natural genetic variation (e.g., reproductive system plasticity or DNA repair mechanisms) among accessions can greatly affect the optimum EMS dosage (Martín et al., 2009). We determined suitable EMS concentrations based on two parameters: 1) the frequency of M1 seed germination, and 2) the frequency of albino chimera M1 seedlings. The frequency of M1 seed germination was greatly reduced with incremental increases in EMS concentrations (Table 1). At EMS concentrations of ≥25 mM, the germination rate was less than 40% (Table 1). Meanwhile, the frequency of albino chimera seedlings increased as EMS concentrations rose (Table 1). A high percentage of albino chimera seedlings were observed from seeds treated with 30 mM (4.0%) and 40 mM EMS (7.2%; Table 1). Although higher EMS concentrations were predicted to induce higher mutation frequencies, this effect could be offset by reduced seed germination and viability (Greene et al., 2003; Kim et al., 2006). Thus, to generate the mutant C24 population, we elected to treat seeds with 25 mM EMS, which produced acceptable germination rates (37.0%) and albino chimeras (1.4%).

From approximately 8,000 of A. thaliana accession C24 seeds treated with 25 mM EMS, 3,620 M1 seedlings were obtained, all of which were used to generate the M2 population. An M2 population with a total of 3,509 individual plants was successfully recovered for use in TILLING. This M2 population also contained 77 partial seed set lines (semi-sterile) and 125 very low seed set lines (sterile). This population including semi-sterile and sterile phenotypes represents a valuable genetic resource for use in forward genetic screens to isolate novel genes affecting reproduction. Each M2 plant sampled for DNA in TILLING was originally isolated from an individual M1 plant to ensure independence of the mutations within the population. Finally, DNA from M2 plants and M3 seeds from 3,509 lines were stored for TILLING analysis. We designated this population of mutant lines the C24TILL collection.

**Detection of EMS mutations from the C24TILL collection** To evaluate the quality of the C24TILL collection, four genes namely *DUO1* (At3g60460), *EXO70C2* (At5g13990), *EXO70H2* (At2g39380) and *APK1b* (At2g28930) were tentatively selected for TILLING analysis (Table 2). These selected genes either had no reported T-DNA lines available (Borg et al., 2011; Li et al., 2010) or were thought to be involved in the self-incompatibility (SI) signaling pathway (Takayama and Isogai, 2005; Kakita et al., 2007), which requires the C24 genetic background for functional analysis. From a total of 7,844 bp fragments screened, 73 mutations were observed.

| Table 1. Dose effects of EMS mutagenesis in A. thaliana C24 M1 plants |
|------------------------|----------------|----------------|----------------|----------------|
| Phenotype              | EMS concentration (mM) | 10 | 25 | 30 | 40 |
| Seed germination rate (%)* | 84.0% | 37.0% | 35.5% | 33.5% |
| Albino chimera seedling (%)* | 0% | 1.4% | 4.0% | 7.2% |
| *Data were recorded on 600 seed batches for each treatment. |

| Table 2. Mutations in 4 selected genes found by TILLING in C24TILL collection |
|-------------------------------|----------------|----------------|----------------|----------------|----------------|
| Gene | Amplicon length (bp) | Number of screened lines | Total screened length (kb) | Number of obtained mutation | *Mutation density (kb–1) |
|      |          |                  |                           | Total | Missense | Sense | Nonsense | Intronic |
| *DUO1* | At3g60460 | 1436 | 3072 | 4289 | 7 | 2 | 4 | 1 | 0 | 1/613 |
| *EXO70C2* | At5g13990 | 2076 | 3456 | 7036 | 29 | 22 | 7 | 0 | 0 | 1/243 |
| *EXO70H2* | At2g39380 | 2127 | 3456 | 6651 | 28 | 21 | 7 | 0 | 0 | 1/739 |
| *APK1b* | At2g28930 | 2205 | 3072 | 7213 | 9 | 3 | 2 | 0 | 4 | 1/345 |
| Total | 7844 | 13056 | 25188 | 73 | 48 | 20 | 1 | 4 |

*Mutation density (kb–1) was estimated after subtracting 40 bp of the length of universal primers for overall gene fragment screened.
Fig. 1. Seven point mutations were found in the DUO1 gene fragment. (A) Diagram showing the analysed DUO1 gene fragment and the location of detected mutations (black arrowheads). Forward and reverse primer sites for TILLING analysis are also indicated (red arrows). (B) Detailed analysis of mutations found in the DUO1 gene fragment. SIFT: Sorting Intolerant From Tolerant program.

| Position from ATG | Exon/Intron | Amino acid changed | SIFT score | Mutation type |
|-------------------|-------------|--------------------|------------|---------------|
| 1 G159A (DUO1_159) | Exon | W53X | 0.00 | Nonsense |
| 2 G641A (DUO1_641) | Exon | R214S | 1.00 | Missense |
| 3 C678T | Exon | - | - | Sense |
| 4 C687T | Exon | - | - | Sense |
| 5 C782T (DUO1_782) | Exon | R261I | 1.00 | Missense |
| 6 G966A | Exon | - | - | Sense |
| 7 G972A | Exon | - | - | Sense |

Fig. 2. Functional characterization of DUO1 mutants. (A) DAPI staining of DUO1 mutant pollen. The DAPI-stained vegetative cell nucleus (white arrowheads) and sperm cell nuclei (white arrows) are indicated. A single larger diploid sperm cell nucleus is designated with an asterisk. Bars: 10 μm. (B) Length of DUO1 mutant siliques. (C) Seed set analysis of DUO1 mutants. Aborted ovules are indicated (black arrowheads). Bars: 1 mm.
obtained (Table 2). These were verified by sequencing and comprised 69.6% missense, 26.0% sense, and 1.4% nonsense (premature stop codon) mutations. Of these, 29 mutations (~30%) were homozygous, fulfilling the expected 1:2 proportion for homozygous/heterozygous M2 plants. This result also suggested that detection of heterozygotes relative to homozygotes was not noticeably compromised by pooling (Greene et al., 2003). It has been reported that EMS predominantly induces G/C to A/T transitions, with guanine residues being the central target of alkylation producing O6-ethylguanine, which produces A/T transitions, with guanine residues being the central target of alkylation producing O6-ethylguanine, which is a target for EMS. A/T transitions, with guanine residues being the central target of alkylation producing O6-ethylguanine, which is a target for EMS.

Accordingly, 98.4% of the mutations in our study were G/C to A/T substitutions, with 62.2% of changes at G and 36.2% at C on the coding strand. In order to estimate the average density of detected mutations per line, we calculated the mutation density according to the following formula: size of fragment screened × number of plants screened/number of identified mutants. The average mutation density was calculated after subtracting 40 base pairs (derived from the common primers) from each gene (Table 2). Based on the four candidate genes screened, we estimated the average mutation density in the C24TILL collection to be 1/345 kb. By contrast, the estimated 1/89 kb mutation density obtained in the Ler collection (Martín et al., 2009) remains as the highest mutation rate reported to date for Arabidopsis thaliana TILLING resources. The mutation density differences between the TILLING populations of A. thaliana might be due to natural genetic variation between accessions for their tolerance to chemically induced mutations. A higher GC content in the analyzed fragment (41%) as compared to the genome average (35%) can also induce a bias in mutation density estimation using the TILLING method (Martín et al., 2009; AGI, 2000). Nonetheless, we have successfully established a new TILLING platform with a sufficient amount of mutations to enable functional genomics studies in A. thaliana accession C24.

**Functional characterization of DUO1 mutants** We further characterized the mutants obtained in the DUO POLLEN 1 (DUO1) gene. DUO1 is a male germline specific essential MYB transcription factor identified in two separate genetic screens (Park et al., 1998; Rotman et al., 2003). Previously described truncation mutations in DUO1, duo1-1 (C to T nonsense mutation at nucleotide 812) from the No-0 accession, and duo1-2 (14 bp insertion at nucleotide 672) from the C24 accession, resulted in a single larger diploid sperm cell that is unable to undergo fertilization (Rotman et al., 2005). Because DUO1 is an essential gene for sperm cell speciation, a homozygous mutant line has never been obtained. Further studies on DUO1 function will require partial loss of function mutants. In the TILLING analysis of the C24TILL collection, we obtained a total of seven mutations within the 1,436 bp DUO1 gene fragment (Fig. 1A). Of these, one was nonsense, two were missense, and the remaining were silent mutations (Fig. 1B). A mutation from G to A at nucleotide 159 created a premature stop codon in DUO1, and we tentatively named this line DUO1_159. The other characterized lines included two missense mutations named DUO1_641 and DUO1_782, in which Arg residues at nucleotides 214 and 261 were substituted with Ile and Ser, respectively.

The only heterozygous line obtained was DUO1_159, which had a shorter silique (average length, 7.8 mm; cf. 12.7 mm in wild type; Fig. 2B). In the siliques of DUO1_159, about half of the ovules were not fertilized and were aborted. Consistent with the previous analysis of duo1-1 and duo1-2 (Rotman et al., 2005), this abortion phenotype was dependent on pollen-related defects. In the heterozygous DUO1_159, approximately half of the pollen grains were shown to have a single diploid sperm cell that was unable to fertilize (Fig. 2A). In contrast to DUO1_159, two missense lines, DUO1_641 and DUO1_782, produced homozygous lines with normal siliques that were indistinguishable from wild type (Fig. 2, B and C). The process of sperm cell specification also looked normal in these missense lines, which produced tricellular pollen with two intensely staining sperm cell nuclei similar to wild type (Fig. 2A). These observations suggested that the mutations in these missense lines did not affect DUO1 function as related to cell specification. This is not unexpected as the point mutations in both lines that caused altered amino acid sequences did not significantly impact the protein function as predicted by SIFT (Sorting Intolerant From Tolerant) program, which predicts whether an amino acid substitution will affect protein function that potentially alter the phenotype (Ng and Henikoff, 2003). SIFT scores less than 0.05 indicated that the amino acid substitution was likely to affect protein function (Ng and Henikoff, 2003), while both missense lines showed SIFT scores of 1.00 with no predicted deleterious impact on the protein function. Although we did not obtain leaky mutants with partial loss of DUO1 function in this screen, our data demonstrated that an array of mutations could be identified for targeted gene fragments from the newly established C24TILL collection.

**CONCLUSIONS**

We have established a new reverse genetic tool for A. thaliana accession C24 by generating an EMS mutagenized population, designated the C24TILL collection, for use in combination with the TILLING screening method.
The development of the C24TILL collection described here represents the third TILLING resource reported for A. thaliana to date (Table 3). TILLING for selected genes from this new collection successfully identified allelic series of induced point mutations, including sense, missense and nonsense mutations. The current C24TILL collection will enable deeper functional genetic studies, especially for researchers aiming to understand novel gene functions in this specific genetic background. Finally, we hope that the established C24TILL collection will serve as a valuable complementary tool alongside existing reverse genetic tools to allow a better understanding of gene functions in Arabidopsis.

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