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# Development of EMS-induced Mutagenized Groundnut Population and Discovery of Point Mutations in the *ahFAD2* and *Ara h 1* Genes by TILLING

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**Abstract: Reducing allergenicity and increasing oleic content are important goals in groundnut breeding studies. *Ara h 1* is a major allergen gene and Delta(12)-fatty-acid desaturase (*FAD2*) is responsible for converting oleic into linoleic acid. These genes have homoeologues with one copy in each subgenome, identified as *Ara h 1.01*, *Ara h 1.02*, *ahFAD2A* and *ahFAD2B* in tetraploid groundnut. To alter functional properties of these genes we have generated an Ethyl Methane Sulfonate (EMS) induced mutant population to be used in Targeting Induced Local Lesions in Genomes (TILLING) approach. Seeds were exposed to two EMS concentrations and the germination rates were calculated as 90.1% (1353 plants) for 0.4% and 60.4% (906 plants) for 1.2% EMS concentrations in the $M_1$ generation. Among the 1541 $M_2$ mutants, 768 were analyzed by TILLING using four homoeologous genes. Two heterozygous mutations were identified in the *ahFAD2B* and *ahFAD2A* gene regions from 1.2% and 0.4% EMS-treated populations, respectively. The mutation in *ahFAD2B* resulted in an amino acid change, which was serine to threonine predicted to be tolerated according to SIFT analysis. The other mutation causing amino acid change, glycine to aspartic acid was predicted to affect protein function in *ahFAD2A*. No mutations were detected in *Ara h 1.01* and *Ara h 1.02* for both EMS-treatments after sequencing. We estimated the overall mutation rate to be 1 mutation every 2139 kb. The mutation frequencies were also 1/317 kb for *ahFAD2A* in 0.4% EMS and 1/466 kb for *ahFAD2B* in 1.2% EMS treatments. The results demonstrated that TILLING is a powerful tool to interfere with gene function in crops and the mutagenized population developed in this study can be used as an efficient reverse genetics tool for groundnut improvement and functional genomics.**

**Key words:** mutation, peanut, oleic acid, reverse genetics

## 1 Introduction

The groundnut (*Arachis hypogaea* L.) also known as peanut, is an allotetraploid species (2n = 4x = 40), belongs to the genus *Arachis*, family Fabaceae[1]. This highly demanded crop produced on 28.5 million ha with a total production of 45.9 million tonnes[2] and widely grown warm temperate and tropical areas between 40° N and 40° S latitudes. Its seed is rich in oil (45–56%), protein (22–30%)[3] and many beneficial nutrients such as minerals, folic acid, niacin and antioxidants[4]. The cake after oil extraction is protein-rich meal for livestock and haulms are significant source of high-quality animal fodder[5]. About 53% of world production is crushed for vegetable oil, 32% is used for confectionery consumption, and the remaining is used for animal feed and seed for production[6]. The health benefits of groundnut also reported about reducing risk of coronary heart disease[7] and anticancer activity[8].

The groundnut oil includes oleic and linoleic acids accounting for about 80% of total fatty acids[9, 10]. In the last decade, breeding studies on this crop have been focused on to increase oleic acid because it provides higher oxidative stability to the oil than linoleic acid[11]. In addition, the food products which contain high oleic groundnut is less prone to oxidation enabling longer shelf life. The positive contribution of high oleic groundnut to general health was reported by different researchers[12−14]. In groundnut, the wild type homoeologous genes (*ahFAD2A* and *ahFAD2B*) encode the delta-12-desaturase (oleoyl-PC desaturase)[15, 16] which catalyzes the conversion of oleic acid to linoleic with the addition of a second double bond into the hydrocarbon

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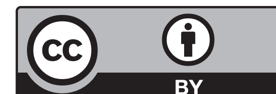

chain of the former[17]. The mutations in *ahFAD2A* and *ahFAD2B* cause to loss of function of oleoyl-PC desaturase activity which is responsible for high oleic acid content[15, 18−20]. Thanks to functional mutations in these genes, the different high oleic varieties were developed[21−23] and later registered[24−26].

The eliminating allergenic effect is other important breeding target in groundnut. Because this crop is in charge of the most food-related allergies related with fatal food-induced anaphylaxis[27]. Among the allergenic proteins detected in groundnut, *Ara h 1* (vicilin) is composed of 12-16% of total seed protein[28] and it is identified by serum IgE from >90% of groundnut-sensitive patients[29]. Two homologues of *Ara h 1* have been identified as *Ara h 1.01* and *Ara h 1.02* in tetraploid groundnut and different studies have been conducted to knock out of these genes for preventing the allergic potential of this crop[30−32].

Reverse genetics is a potentially important approach to identify novel mutations in gene of interest. TILLING, one of the reverse genetics methods, can be applied to any plant species, regardless of its genomic structure and ploidy level[33]. This non-transgenic method does not require transformation compared to other reverse genetic methods such RNAi technology[34] and T-DNA insertion mutagenesis[35]. TILLING aims to find nucleotide changes induced by chemical mutagenesis in target genes[36, 37] which make it possible to modify the protein function. EMS (ethyl methane sulfonate) is widely preferred mutagen in this strategy because it induces single nucleotide alterations by alkylation of specific nucleotides causing wide spectrum mutations[38, 39]. This can be silent, nonsense, missense and splicing mutations in gene coding regions[40, 41]. TILLING was successfully applied to different plant species such as arabidopsis[42]; oat[40]; canola[43]; soybean[44−46]; rice[47]; wheat[48]; groundnut[30, 31]; tomato[49], sunflower[50] and tobacco[51] showed that this method is an important alternative for functional analysis in plant species. From this perspective, we report to development of EMS induced groundnut mutant population and TILLING analysis in $M_2$ generation using four homologues genes (*Ara h 1.01*, *Ara h 1.02*, *FAD2A* and *FAD2B*) to discover point mutations.

## 2 Experimental Procedures

### 2.1 Plant material

The groundnut cultivar, NC-7 (Virginia market type), belongs to subsp. *hypogaea* var. *hypogaea*. It has good agronomic traits such as large pods, high yielding and shelling percentage[52]. The seeds of this cultivar were obtained from the West Mediterranean Agricultural Research Institute of Turkey. Before the mutagen applications, the original seeds were grown in two generations to ensure homogeneity.

### 2.2 Mutagenesis

Approximately 3000 seeds were imbibed in tap water for 10 hours. These seeds were then transferred to aqueous solution of mutagen. Two different concentrations of EMS, 0.4% and 1.2%, were used for TILLING. The 1500 seeds were soaked in 0.4% (v/v) EMS solution at the ratio of 75 seeds/100 mL and shaken gently with 10 h at room temperature. In the other application, remaining 1500 seeds were soaked with agitation (2.5 hours on shaker) in 2000 mL of distilled water containing 1.2% (v/v) EMS concentration. After these treatments, seeds were thoroughly washed with deionized water for three times and rinsed extensively in running water overnight. The EMS-treated seeds ($M_1$) were sown in the experimental field with an inter-row spacing of 70 cm and an intra-row spacing of 20 cm. $M_2$ seeds were collected from individual $M_1$ plants and one pod from each $M_2$ seeds was planted in greenhouse and later thinned to have a single plant per pot. $M_3$ seeds from each $M_2$ plant were packed and stored for further studies.

### 2.3 DNA isolation and pooling

Total DNA was isolated with a modified CTAB method[53] using leaf tissue from individual $M_2$ plants. All genomic DNAs were quantified on a 1.0% agarose gel using lambda DNA and normalized. The equivalent amounts of DNA from individual $M_2$ plants were pooled four-fold in 96-well format.

### 2.4 Primers and amplification of studied genes

The homologues of *Ara h* and *FAD2* genes were selected to conduct TILLING study. Primer sets for these genes (*Ara h 1.01*, *Ara h 1.02*, *FAD2B* and *FAD2A*) were previously designed by Chu *et al.*[54] and Guo *et al.*[31]. The product sizes and primers used for the amplification were listed in Table 1.

The target regions for *FAD2* genes were amplified on a Thermo Scientific Arktik Thermal Cycler (Vantaa, Finland) using 96-well plates and carried out in a 20 μL volume consisting 1.5 μL of 10X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 μL each of forward and reverse primers, 5 unit of *Taq* DNA polymerase (Thermo Fischer Scientific), 1.5 μL of genomic DNA template and Milli-Q water to make up the final volume. The thermocycling condition was 95℃ for five minutes for initial denaturing, followed by 30 cycles of 95℃ for 30 sec, 48℃ for 30 sec, 72℃ for one minute, one cycle of 72℃ for seven minutes and 4℃ hold for storage. The PCR protocol for amplification of *Ara h 1.01* and *Ara h 1.02* genes was as follow: 1.3 μL of 10X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 μL each of forward and reverse primers, 5 unit of *Taq* DNA polymerase (Thermo Fischer Scientific), 1.5 μL of genomic DNA and 14.1 μL deionized water. PCR parameters: One cycle of 98℃ for 30 sec followed by 35 cycles of 98℃ for 10 seconds, 58.7℃ (*Ara h 1.01*)-59.6 ℃ (*Ara h 1.02*) for

Table 1 The primer pairs used to amplify target loci.

| Genes | Product size (bp) | Forward primer | Sequence (5'-3') | Reverse Primer | Sequence (5'-3') | Reference |
|---|---|---|---|---|---|---|
| *ara h 1.01* | 1.865 | 1306 | GAGCAATGAGAGGGAGGGTT | 2079 | TCTTCGTCTTCGTCCTCCTCTTCTT | Guo *et al.* (2015)[31] |
| *ara h 1.02* | 1.666 | 1306 | GAGCAATGAGAGGGAGGGTT | 1309 | CCTCCTCTTCTTCCCACTCTTG | |
| *ah FAD2A* | 826 | aF19 | GATTACTGATTATTGACTT | 1056R | CCAACCCAAACCTTTCAGAG | Chu *et al.* (2011)[54] |
| *ah FAD2B* | 1.214 | bF19 | CAGAACCATTAGCTTTG | R1/FAD | CTCTGACTATGCATCAG | |

one minute, and 72℃ for 1.5 minutes. The last steps in the PCR was 72℃ for 10 minutes and held at 4℃. All amplified products in this study were separated on 2.5% agarose gels. Heteroduplex formation was performed after PCR with the steps; denaturation at 95℃ for 3 minutes, followed by 70 cycles of 70℃ for 20 sec decreasing by 0.3℃ per cycle with a final held at 8℃.

### 2.5 Mutation discovery by TILLING

TILLING process was conducted with use of mutation discovery kit (DNF-910-K1000T) (AATI, Ames, USA) which offers easy, high-throughput and profitable results for mutational screening. In this protocol, the working solution of the dsDNA cleavage enzyme by diluting the enzyme with the T-Digest buffer in the ratio of 1:125 was prepared and only 2 μL of this working enzyme solution was added for each sample well after heteroduplex formation step. This was followed by incubation of 96-well plates at 45℃ for 45 minutes and then 20 μL of the dilution buffer added to each well. These samples were analyzed in the Fragment Analyzer™ which was automated capillary electrophoresis (Fragment Analyzer™, Advanced Analytical Technologies GmbH, Heidelberg, Germany). In this capillary system, solutions for the mutation discovery kit (DNF-910-K1000T) were used to visualize digested products ranging from 35 to 5000 bp. Raw data were analyzed using PROSize™ software (Version 1.2.1.1) (Advanced Analytical Technologies, AMES, IA, USA). To confirm a potential mutant from the pool, individual genomic DNAs were used to PCR amplification with specific primers for the target gene. Before sequencing, PCR products were purified using the GeneJET Gel Extraction Kit (Thermo) according to the manufacturer's instructions. The amplicons were sequenced by Macrogen (China) and sequence analysis was performed in MEGA software version 5[55]. The sequences having mutations were translated to protein sequence with the online source https://web.expasy.org/translate/. The online software SIFT (Sorting Intolerant from Tolerant) (https://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html) was used to predict whether an amino acid substitution affects protein function[56]. SIFT score was ranged from 0 to 1 and the amino acid substitution is predicted damaging is the score is ≦0.05, and tolerated if the score is >0.05.

## 3 Results

### 3.1 Development of TILLING population

A large seeded cultivar, NC-7, was used for generating TILLING population. Two different EMS mutagenesis treatments, 1.2% for 2.5 h and 0.4% for 10 h, were tested on 3000 seeds in this study. The germination rates were calculated as 90.1% (1353 plants) for 0.4% and 60.4% (906 plants) for 1.2% EMS concentrations in $M_1$ generation. The lethality of the groundnut population was therefore about 24.7% under mutagen application. The common observed developmental deformation was related to cotyledon morphology (Fig. 1) and altered phenotype was under 1% in comparison with the wild-type. $M_2$ seeds were harvested from individual $M_1$ plants however 68.7% (930) and 67.4% (611) of the $M_1$ plants produced seeds in 0.4% and 1.2% EMS doses, respectively. Among all the fertile plants (1541), 384 seeds from each EMS mutagenesis treatments were advanced for $M_2$ plants production in greenhouse and DNA was isolated from leaf tissues of those plants for PCR analysis.

### 3.2 Identification of mutations

The single copy genes, *Ara h 1.01*, *Ara h 1.02*, *FAD2B* and *FAD2A*, were selected to identify mutations in TILLING study. The total amplified region length of these genes was about 5571 bp (Table 1) and each EMS-treated $M_2$ plant was screened in that frame. After heteroduplex PCR and digestion with enzyme, Fragment Analyzer™ detected potential mutations in eight pooled wells of plates for four genes and these 32 individuals were sequenced (data not shown). Among them, two induced mutations were confirmed in *FAD2B* and *FAD2A* gene regions (Fig. 2) from 1.2% and 0.4% EMS-treated populations, respectively. DNA from $M_2$ plant coded 339 contained a heterozygous SNP (T→A) (position 993 in the *FAD2B* gene sequence) (Table S1) resulting in an amino acid change, Serine to Threonine, compared to wild type sequence (Table S2). This substitution was predicted to be tolerated with a score of 0.28 according to SIFT analysis. Similarly, the other mutation was heterozygous SNP (G→A) (position 861 in the *FAD2A* gene sequence) (Table S1) and causing amino acid change, Glycine (G) to Aspartic acid (D) (Table S2) in DNA from $M_2$ plant coded 547. SIFT analysis showing this substitution from G to D was predicted to affect protein function with a score of 0.01. These identified point mutations

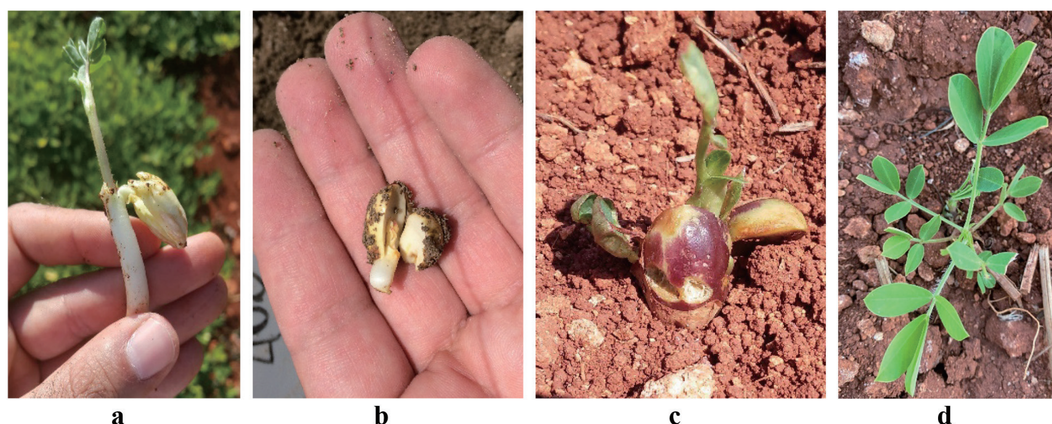


Fig. 1 a, b, c) EMS through induced mutation leading to deformation and retardation in the developmental stages of groundnut. d) wild type.

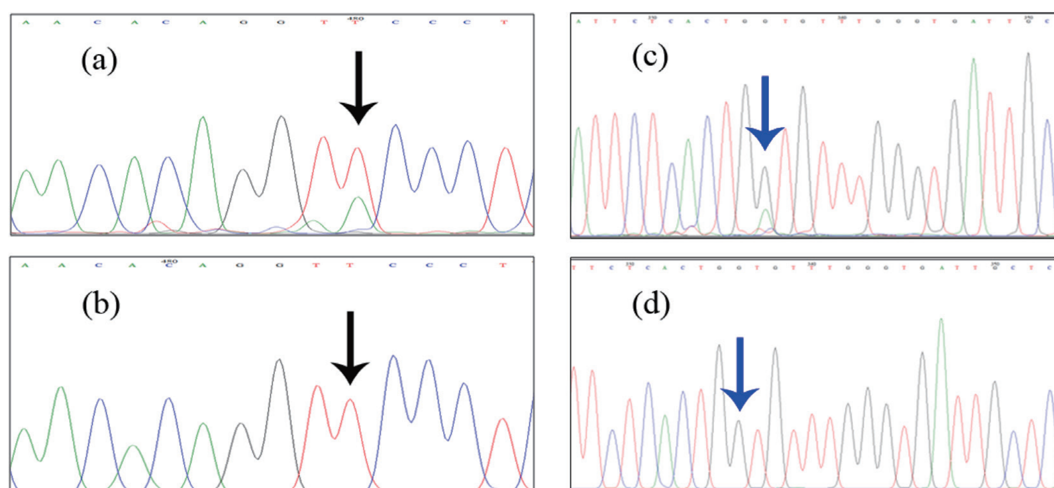


Fig. 2 Locations of *FAD2B* and *FAD2A* mutations in the lines 339 and 547, respectively. DNA from line 339 (single $M_2$ plant) and NC-7 (control) were amplified with the primer pair of bF19-R1/FAD for *FAD2B* and partial sequence traces were shown with double peak at the positions of nucleotide in line 339 (a) and single peak at same position in NC-7 (b). The primer pairs of aF19-1056R amplified DNA from 547 (single $M_2$ plant) and NC-7 (control) for *FAD2A*. The partial sequence showed double and single peaks for line 547 (c) and NC-7 (control) (d) at the same position, respectively.

indicated that nucleotide changes were one transversion (T→A) and one transition (G→A) in our TILLING study. After heteroduplex formation, the dsDNA enzyme cleaved the fragments with mutations (Figs. 3 and 4). The cleaved bands corresponding to the induced mutation were located about 470 bp and 900 bp of amplified products for *FA2DB* gene (Fig. 3). The lengths of bands were 340 and 530 bp for the cleaved fragments formed by digestion in mutation point for amplified product of *FAD2A* gene (Fig. 4). No mutations were detected in *Ara h 1.01* and *Ara h 1.02* for both EMS-treatments after sequencing. Mutation frequency was calculated as the total number of confirmed mutations divided by the total number of base pairs screened[31]. The average mutation frequency was estimated to be one mutation per 1566 kb for *FAD* genes considering two EMS applications together. The mutation rates were 1/317 kb for *FAD2A* in 0.4% EMS (12 h) and 1/466 kb for *FAD2B* in 1.2% (2 h), respectively. Based on the mutation frequency in the four targeted genes (Table 1), we estimated the overall mutation rate to 1 mutation every 2139 kb (2 mutations in 5571 bp of DNA from the 768 $M_2$ plants screened).

## 4 Discussion

TILLING is a flexible method for modifying gene functions by mutations which possibly affect protein function which might cause to partial phenotypic change or expression difference in the target gene[57] without involving transgenic modification. The optimization of mutagenesis is the key factor for the success in this method because of the toxicity and sterility on germinal tissue[58]. In groundnut,

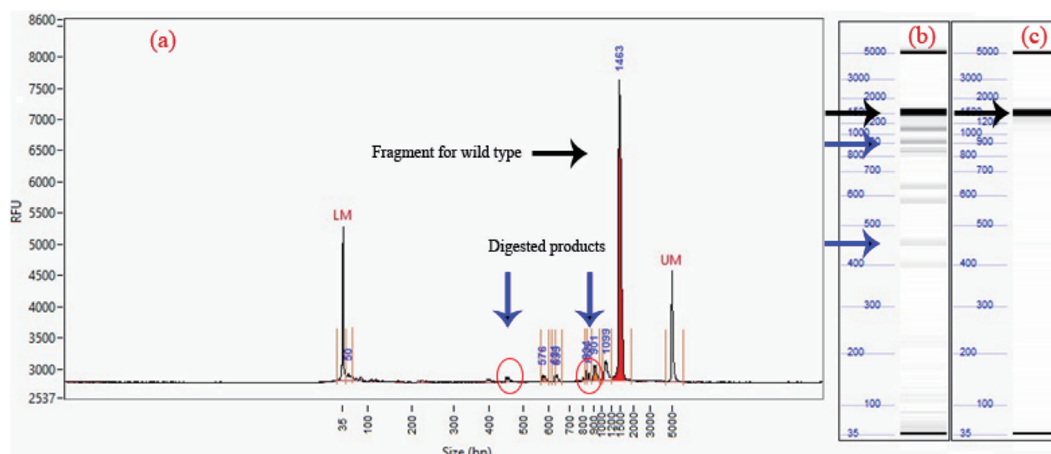


Fig. 3 The amplified *FAD2B* gene region was monitored in ProSize™ software. Blue arrows were cleavage product sizes as peak (a) and fragment (b) from four pooled DNA included lines 339, 340, 341 and 342. Wild type (c) had the only expected amplified product (black arrow). LM is lower marker (35 bp) and UM is upper marker (5000 bp).

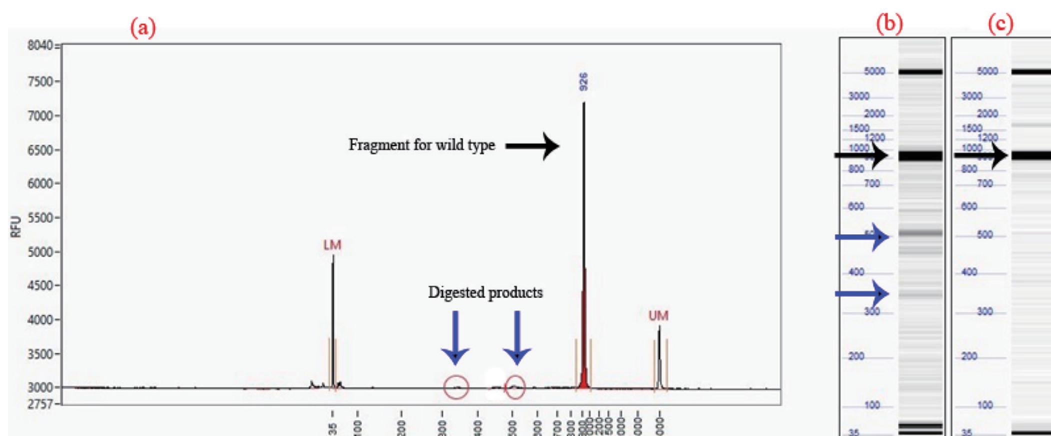


Fig. 4 The amplified *FAD2A* gene region was monitored in ProSize™ software. Blue arrows were cleavage product sizes as peak (a) and fragment (b) from four pooled DNA included lines 546, 547, 548 and 549. Wild type (c) had the only expected amplified product (black arrow). LM is lower marker (35 bp) and UM is upper marker (5000 bp).

two EMS mutagenesis treatments (1.2% and 0.4%) were applied to seeds by Knoll *et al.*[30] who monitored high germination frequencies and detected many SNP mutations in fatty acid desaturase and major allergen genes. Guo *et al.*[31] also captured nucleotide changes with the application of 0.4% EMS mutagen on this crop. These tested EMS doses were therefore selected in the present investigation without preliminary experiments to produce mutagenized populations. Although 1.2% EMS concentration treatment is detrimental to the survival of the different plant species such as arabidopsis[38] and rice[47], we obtained about 60% germination frequency in that mutagen dose. The cultivated groundnut has a polyploid genome and this ploidy level can provide tolerance to higher mutation dose because of genetic buffering[59], similar to that observed in polyploid mutant populations of wheat[57, 60]. The polyploid genomic structure might also affect the observed phenotypic mutation rate which was <1% in our study and similar low frequency was previously described in groundnut[30] and also other polyploid crops such as wheat[57].

Based on the mutation frequency in all the studied genes (*Ara h 1.01*, *Ara h 1.02*, *FAD2A* and *FAD2B*), we estimated the overall mutation rate as 1/2139 kb for each EMS applications. This value was found to be higher than in TILLING population of barley *Hordeum vulgare* L. (1/2500 kb)[61] and lower when compared to other populations in sorghum (1/526 kb)[62], rice (1/294 kb)[47], sunflower (1/475 kb)[50], rice (1/1000 kb)[63] and *Aegilops tauschii* (1/77 kb)[64]. The difference in mutation density might be sourced of various factors such as species, type of mutagen, treatment procedure, mutagen concentration and treated organs[62, 65]. Based on the single gene, mutation densities were 1/317 kb for *FAD2A* and 1/466 for *FAD2B* genes in the 0.4% and 1.2% EMS applications, respectively. Although groundnut seeds were exposed with same concentration of EMS, different mutation rates were observed in the study conduct-

ed by Knoll *et al.*[30] who reported lower mutation frequency was 1/501 kb for *FAD2A* in 0.4% EMS application and not captured any mutations for *FAD2A* and *FAD2B* in 1.2% EMS dose. Knoll *et al.*[30] and Guo *et al.*[31] also recorded higher overall mutation frequencies on groundnut compared with our result in 0.4% EMS application. The mutation detection method might be one of the reasons for the variable values in mutation rates. Because our TILLING process was conducted with use of mutation discovery kit (DNF-910-K1000T) (AATI, Ames, USA) differently from the studies were carried out by Knoll *et al.*[30] and Guo *et al.*[31] used CEL I/LI-COR and TILLING by sequencing approaches, respectively. The effect of environmental conditions on the plant response[44] and difference in the selected genotype[62] might also be other reasons. The point mutations in our tilling study indicated that nucleotide changes were one transversion (T→A) and one transition (G→A) and both of types were observed in TILLING populations of rice[47], tomato[49] and cucumber[41].

In groundnut, delta-12-desaturase (oleoyl-PC desaturase) converts oleic into linoleic acid and it is coded by two homologous genes *ahFAD2A* and *ahFAD2B*[15, 18]. The repression of these two genes makes possible higher oleic content with changes G to A transition at the 448 position of the coding region of *ahFAD2A*[66], and "A" insertion in 441_442 position in the coding region of *ahFAD2B*[15, 20]. This missense mutation in *ahFAD2A* caused to aspartic acid to asparagine transition at position 150[20]. The cultivar, NC-7 (subsp. *hypogaea* var. *hypogaea*) used in this study had this spontaneous mutation[52] which was frequent among subspecies *hypogaea* accessions[67]. A new mutation was therefore identified at position 861 causing glycine to aspartic acid transition in the present investigation. The 'protein function was predicted to be affected by this amino acid change according to SIFT analysis. Similarly, Knoll *et al.*[30] pointed out that the most of the nucleotide changes in *ahFAD2A* gene causing aspartic acid transition in groundnut TILLING population treated with 0.4% EMS. In homologue gene *ahFAD2B*, insertions cause to severe stop codons which leading to high oleic ratio[15, 20, 68]. Differently from this type mutation, the nucleotide change captured at position 993 in the coding region of *ahFAD2B* resulting in amino acid change, serine to threonine which was rare transition in that gene compared other studies in groundnut conducted by Knoll *et al.*[30], Fang *et al.*[21] and Guo *et al.*[31]. Differently from common mutations in *ahFAD2B*, Nadaf *et al.*[23] developed high oleic mutants had novel SNP changes caused to amino acid transitions, one at 362 from glutamate to glycine, and another at 372 from glycine to serine. These different mutations in *ahFAD2B* showed that this gene functions can be altered with various SNP changes lead to high oleic content. The TILLING method is therefore effective approach to generate high oleic mutants that may be directly used in commercial activities. The other homologous genes, *Ara h 1.01* and *Ara h 1.02*, were also screened in the present TILLING research however there was no SNP changes in both EMS mutagenesis treatments. The number of screened plants (768) in our study might be reason for this result because Knoll *et al.*[30] identified five mutations among the 3420 plants in similar EMS doses. This showed that higher mutation frequencies and greater allelic variation should be obtained with the increase the size of the required population in TILLING population[69]. On the other hand, the number of detected mutations in the same gene might be affected by enzyme activity and probability of mutations presenting in the effective region[60, 70]. Not only population size and the mutation frequency, but also the method used to identify specific mutations in the population is equally important for successful TILLING applications[40]. Up to date, different techniques have been selected to capture mutations in the studies conducted by Greene *et al.*[71], Cooper *et al.*[44]; Uauy *et al.*[60]; Tindall *et al.*[72]; Gilchrist *et al.*[43]; Mascher *et al.*[73]. The mutation discovery kit (AATI, Ames, USA) and a detection platform (Fragment Analyzer™) was used to detect SNP changes in the present TILLING approach. In this technology, mutation detection times are cut in half because of the detection system's speed and streamlined methods used with it compared to gel systems. Identification multiple cuts in one gene, no clean up step and eliminating use of labeled primer sets are also important features of this setup. It also examines fragments up to 10,000 base pairs and has user-friendly software to screen mutations. These are significant advantages compare to CEL I/LI-COR heteroduplex detection method which requires labeled primers in PCR reactions[58] and also analysis of TILLING gel images is difficult[71]. In addition, Fragment Analyzer™ has faster electrophoresis run times than other mutation detection method, Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF)[40]. Although these features and benefits, we faced some drawbacks with mutation discovery kit used in this study. The first problem was undesirable peaks and fragments (Fig. 3) which were monitored in software gave rise to false SNPs. We observed cleaved fragments and peaks in eight wells, however, the right SNPs were detected only two of them after sequencing. The residues of main and digested fragments might generate these unexpected results. The other issue was peak size. Main fragment and cleaved products were observed together in output graphic and this issue caused to lower peak size (volume) for cleaved fragments resulted with uncertainty to identify mutations, for example Fig. 4. To overcome these detection problems, we sequenced potential mutant DNAs to confirm nucleotide changes and similarly, identified mutations with the mutation discovery kit were later checked by Sanger sequencing in the study conducted by Mascher *et al.*[73]. With respect to economical side, the mutation discovery kit

is costly and needs to initial investment in terms of detection platform like denaturing high-performance liquid chromatography (DHPLC) method[74]. The sequencing-based method of TILLING seems to better approach to detect mutations because it does not require specific mutation-detection platform. It can be used for direct mutation determination without any pre-screening[33] and would be cost-friendly with the pooling large numbers of samples[45] with the high sensitivity compared to CEL I/LI-COR[31] and Mutation discovery kit/Fragment Analyzer. It also eliminates disadvantage of CEL I enzyme which especially recognizes certain mismatches might decrease the sensitivity[75].

## Author Contributions

Engin Yol performed research, analyzed data, and wrote the original manuscript. Merve Başak, Sibel Kizil and Kürşat Karaman conducted field trails and laboratory studies. Bülent Uzun supervised the research design and reviewed the article. All authors read and approved the article.

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## Conflict of Interest

The authors declare no conflict of interest.

## Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21075

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