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Phenotypic Characterization of Point Mutations Spanning FHA Domain and C-terminal Region of *Dawdle* Gene in *Arabidopsis*

Arabidopsis'te Dawdle Geninin FHA Alanını ve C-terminal Bölgesini Kapsayan Nokta Mutasyonlarının Fenotipik Karakterizasyonu

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ABSTRACT: The screening analysis of loss-of-function alleles in *Arabidopsis thaliana* revealed a mutation in the At3G20550 gene, called *DAWDLE* (*DDL*). The mutation in the *DDL* gene causes pleiotropic phenotypes and reduced the levels of several microRNAs. The *DAWDLE* gene encodes a protein with a Fork Head-Associated (FHA) domain, found in large range of proteins with significant cellular processes in prokaryotes and eukaryotes. However, it is not completely known whether the FHA domain and C-terminal region of the DDL are necessary for its function. The aim of this study was to determine the function of both regions by conducting a phenotypic analysis of point mutations spanning the FHA domain and C-terminal region in DDL Targeted Induced Local Lesions IN Genome (TILLING) screen was performed in the *Columbia erecta-105* background of *Arabidopsis* resulting in point mutations spanning both regions of *DDL*. The mutants were phenotypically characterized. Height of the plant, hypocotyl and root length, and fertility were measured. Phenotypic analyses of the mutants revealed *ddl* phenotypes of varying degrees in different organs. Reduction in fertility and shortening in root, hypocotyl and stem lengths of the TILLING mutant lines suggest that the FHA domain and C-terminal region may require for *DDL* function in *Arabidopsis*.

Keywords: *Dawdle*, Fork Head-Associated Domain, Targeted Induced Local Lesions in Genome, Ethyl Methane Sulfonate, *Arabidopsis*

ÖZ: *Arabidopsis thaliana*'daki gen fonksiyon kaybı alellerinin analizi, *DAWDLE* (*DDL*) adı verilen At3G20550 geninde bir mutasyonu ortaya çıkarmış ve *DDL* genindeki bu mutasyonun pleiotropik fenotiplere neden olduğu ve birçok mikro RNA seviyesini de düşürdüğü rapor edilmiştir. *DAWDLE* geni, prokaryot ve ökaryotlarda önemli hücresel fonksiyonlara sahip birçok proteinde de bulunan Çatal Başlı-İlişkili Bölge (FHA) alanına sahip bir proteini kodlar. Ancak, DDL proteinin FHA alanının ve C-terminal bölgesinin bu genin işlevi için gerekli olup olmadığı tam olarak bilinmemektedir. Bu çalışmada, *DDL* geninin FHA alanı ve C-terminal bölgesini kapsayan nokta mutasyonlarının fenotipik karakterizasyonu yapılarak her iki bölgenin DDL fonksiyonunda rolü olup olmadığı analiz edilmiştir. *Arabidopsis*'in *Columbia erecta-105* yabanıl tipinde 'Genomda Hedefli İndüklenmiş Lokal Lezyonlar' (TILLING) hat taraması yapılarak *DDL* geninin FHA ve C-terminal bölgesini kapsayan nokta mutasyonları tespit edilmiş, ardından bu nokta mutasyonları taşıyan homozigot mutantlar belirlenerek bitki boyu, hipokotil ve kök uzunluğu ve tohum sayısı seviyesinde fenotipik olarak karakterize edilmiştir. Mutantların fenotipik analizi, farklı bitki organlarında değişen derecelerde benzer *ddl* fenotiplerini ortaya çıkarmıştır. TILLING mutant hatlarının tohum sayısındaki düşüş, kök, hipokotil ve gövde uzunluklarındaki kısalma, *Arabidopsis*'te FHA ve C-terminal bölgesinin *DDL* geninin fonksiyonu için önemli olabileceğini ortaya koymuştur.

Anahtar Kelimeler: *Dawdle*, Çatal Başlı-İlişkili Bölge, Genomda Hedefli İndüklenmiş Lokal Lezyonlar, Etil Metan Sülfonat, *Arabidopsis*

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1. INTRODUCTION

Many eukaryotic and several prokaryotic proteins have been identified and categorized with their phosphobinding domain. Phosphobinding domains typically recognize specific peptide motifs on their binding partners, in a fashion that depends on the phosphorylation of a tyrosine (pTyr), serine (pSer) or threonine (pThr) residues in the recognition sequence. One of the well-characterized phospho-threonine (pThr) binding domains is Fork-Head Associated (FHA) domain (Durocher et al., 1999). FHA is one such protein-protein interaction domain that was initially identified and named as a conserved region of fork-head transcription factor in Drosophila (Kim et al., 2002). Later, FHA domain has been reported to be present in more than 2,000 proteins including protein kinases, DNA-binding transcription factors, RNA-binding proteins, protein phosphatases, kinesins, proteases, glycoproteins and zinc-finger proteins from all kingdoms including bacteria, animals, and plants (Durocher et al., 1999; Hammet et al., 2000; Liao et al., 1999; Sun et al., 1998). Thus, FHA domains are not restricted to fork-head transcription factors (Mahajan et al., 2008). The existence of FHA domains in a large amount of proteins suggests that the proteins with FHA domain play an important role in several cellular processes such as intracellular signal transduction, transcription, protein transport, DNA damage response, cell cycle regulation and protein degradation (Durocher et al., 2000a; Durocher et al., 2000b; Mahajan et al., 2008). FHA domain recognizes specifically pThr residues on targets. This is a unique characteristic of FHA domain compared to other phosphor-binding domains like WW and 14-3-3 that recognize both pThr and pSer residues (Mahajan et al., 2008).

There are 18 genes in the *Arabidopsis* genome that encode predicted proteins that contain an FHA domain. In a screen of several T-DNA mutant populations to isolate loss-of-function alleles in *Arabidopsis* genes that encode a protein with a conserved FHA domain, a mutation in the *At3G20550* gene was isolated. The slow and prolonged growth period of the mutant gave the name *DAWDLE* (*DDL*) to this gene. *DDL* is located on chromosome 3 of *Arabidopsis*, has 10 exons, and code for a protein of 314 amino acids (Supplementary Figure 1 and 2).

DDL is a nuclear localized protein consisting of FHA domain and arginine-rich (Arg-rich) region. The FHA domain of DDL is found near to the C-terminus, and spanning amino acid residues from 218 to 282 (Supplementary Figure 3).

The Arg-rich region of DDL is located near to the N-terminus, and contains several putative nuclear localization signals and RNA binding domain (Morris et al., 2006; Narayanan et al., 2014; Yu et al., 2008). Two independent T-DNA mutant alleles in *ddl* have been isolated and named as *ddl-1* and *ddl-2*. Since both are in the Wassilewskija-2 (Ws-2) genetic background of *A. thaliana* and do not produce *DDL* messenger RNA (mRNA) transcript, *ddl-1* and *ddl-2* are null alleles of *DDL*. *ddl-1* and *ddl-2* mutant plants are developmentally delayed and display pleiotropic phenotypes such as defective roots, shoots and flowers, reduced number of seed and cell division defect in meristematic tissues. T-DNA insertions in *dld-1* and *ddl-2* are located near the intron 3/exon 4 border, and to the upstream of the predicted initiation codon, respectively (Morris et al., 2006). Also, *ddl* knockout mutants have demonstrated a reduced accumulation of small RNAs including several microRNAs, as well (Narayanan et al., 2014; Yu et al., 2008).

Afterwards, to reveal whether FHA domain and Arg-rich region of DDL are required for DDL function, two independent *Arabidopsis* TILLING (Targeting Induced Local Lesions IN Genomes) mutant collections, which have been induced with ethyl methane sulphonate (EMS), and developed in the Columbia (Col) and *Landsberg erecta (Ler) glabrous1-1* genetic background of *A. thaliana*, (Martin et al., 2009; Till, Reynolds, et al., 2003) have been partially screened and characterized (Morris et al., 2006; Narayanan et al., 2014). TILLING is a non-transgenic method based on an endonuclease enzyme activity, which preferentially cleaves mismatches in heteroduplex between wild-type and mutant DNAs, for the direct examination of gene function. Subsequent analysis of cleaved products on an agarose gel allows for the rapid identification of induced point mutations producing weak nonlethal alleles in the genome of *A. thaliana*. M3 seeds obtained from M2 plants are used for phenotypic analysis (Greene et al., 2003; McCallum et al., 2000; Till, Colbert, et al., 2003). One of those TILLING alleles, *ddl-3*, which possesses an amino acid change on residues of the FHA domain, shows the same phenotypes as the null alleles; *ddl-1* and *ddl-2* (Morris et al., 2006). The phenotype of *ddl* mutant also resembles the phenotype of a mutant in *DICER-LIKE1* (*DCL1*) (Schauer et al. 2002). In *Arabidopsis*, DCL1 is involved in processing of pri-microRNA into pre-microRNA, and also catalyses the formation of mature microRNA (miRNA) in the nucleus (Chen, 2005). miRNA is 20 to 24 nucleotide long single stranded RNA that is involved in the regulation of gene expression by cleavage or translational repression of its target messenger RNAs (Bartel, 2004). In *dcl-1*, miRNAs are not processed leading to the absence of mature miRNA and the regulation of gene expression (Kurihara & Watanabe, 2004). To test whether DDL is involved in miRNA synthesis, the levels of several miRNAs have been checked in the *ddl* mutants (*ddl-1*, *ddl-2*, and *ddl-3*). There is a 2.2 to 3-fold reduction in the pri, pre and mature miRNA levels in the mutants compared to the wild type allele (Narayanan et al., 2014; Yu et al., 2008; Zhang et al., 2018). One possible explanation for reduced levels of miRNAs in *ddl* mutants is that DDL regulates the expression of genes involved in miRNA biogenesis. Although it has not been observed that *ddl* mutations have an explicit effect on the expression of genes regulating miRNA processing, it has been shown that DDL interacts with an N-terminal portion of DCL1 and DCL3, which is a DCL1 homolog in charge of small

interfering RNAs (siRNA) production, in Arabidopsis (Yu et al., 2008; Zhang et al., 2018). Furthermore, data have shown that DDL is an RNA-binding protein, and that the N terminal domain of DDL shows conservation with several proteins involved in mRNA metabolism (Yu et al., 2008). Therefore, it was pointed out that DDL globally effects miRNA processing and accumulation, and it is required for siRNA biogenesis (Zhang et al., 2018). However, it was not confirmed yet that DDL binds to specifically to pri-miRNAs *in vitro*. Also, it is not yet comprehensively understood which part of DDL is important and indispensable for its function. In order to understand the function of DDL and the contribution of different domains towards its function, it is necessary to characterize the DAWDLE protein. Therefore, the aim of this study was to identify a few key amino acid residues for a better understanding of the FHA domain and C-terminal region of DDL thorough isolation and characterization of new different TILLING point mutation alleles of *DDL*. This study isolated and phenotypically characterized three different mutants with an amino acid change in the FHA domain and C-terminal region of DDL and showed that this change may be significant for DDL function.

2. MATERIALS and METHODS

2.1. Plant Material and Growth Conditions

Seeds for TILLING mutant lines including 2206, 2317, 2182, 955 and 731 were ordered from the Arabidopsis Biological Resource Center of The Arabidopsis Information Resource (TAIR SeqViewer, 2021), which is online database of genetic and molecular biology data for the model plant *Arabidopsis thaliana*. The TILLING mutations are located in the FHA Domain (TILLING 2206 and 2317) spanning the 218th and 282nd amino acid interval, and the C-terminal region (TILLING 2182, 955 and 731) spanning the 283th and 314th amino acid interval of DDL. In these TILLING 2317, 2206, 955, 2182 and 731 point mutants, Arginine is replaced by Histidine, Glutamic acid by Lysine, Arginine by Lysine, Arginine by Glutamine and Valine by Isoleucine, respectively. Each TILLING mutant line with genetic characteristics (Table 1) was mapped on *DDL* cDNA (Figure 1).

Table 1. TILLING mutant lines studied*

TILLING Lines	Position in Gene	Mutation Wild - Mutant	Functional Effect	Codon Change Wild - Mutant	AA Change Wild - Mutant
2182 (10th Exon)	2869	G → A	Missense Mutation	CGA → CAA	Arg303Gln
2206 (7th Exon)	2386	G → A	Missense Mutation	GAA → AAA	Glu249Lys
2317 (6th Exon)	2192	G → A	Missense Mutation	CGT → CAT	Arg223His
955 (9th Exon)	2674	G → A	Missense Mutation	AGA → AAA	Arg286Lys
731 (10th Exon)	2877	G → A	Missense Mutation	GTA → ATA	Val305Ile

**Each mutant line is numbered as shown in the first column. As second and fourth columns display point mutations of each TILLING line at the nucleotide and codon levels, respectively. Third and fifth column identify the functional effects of those single nucleotide changes on amino acid level and protein structure, respectively. Position 1 in the gene sequence corresponds to the first nucleotide in ATG (start) codon. (AA; amino acid, A; Adenine, G; Guanine, T; Thymine, C; Cytosine, Arg; Arginine, Gln; Glutamine, Glu; Glutamic acid, Lys; Lysine, His; Histidine, Val; Valine, Ile; Isoleucine)*

Blue lines in Figure 1 represent the location of each TILLING mutant line with corresponding exon regions on *DDL* cDNA. As TILLING 2317 and 2206 mutant lines have a point mutation on the exon regions (6 and 7, respectively) coding FHA domain of DDL, TILLING 955, 2182 and 731 mutant lines have a point mutation on the exon regions (9, 10, and 10, respectively) corresponding to C terminus of DDL. (ATG; start codon, bp; base pair).

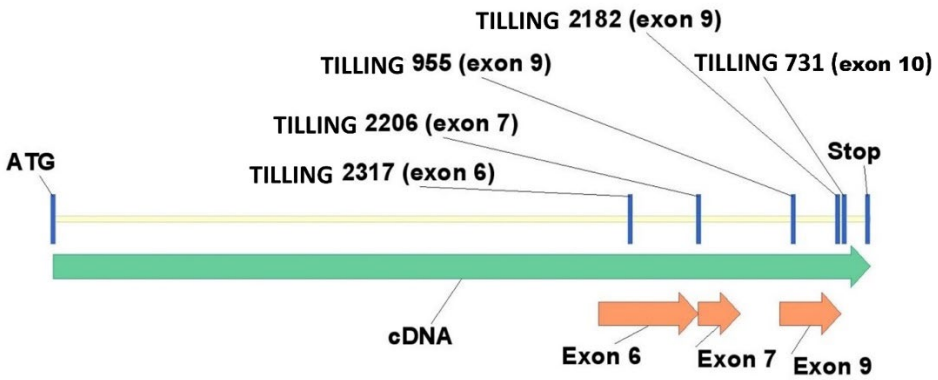

Figure 1. TILLING mutant lines mapped on *DDL* cDNA (946 bp from ATG to Stop)

M3 seeds of Arabidopsis thaliana accessions Ler gl-1, numbered as 2182, 2206, 2317, 955 and 731, were sterilized with the solution of 200 milliliter (ml) liquid bleach (12.5% NaClO) and 3 ml hydrochloric acid (HCl, 6.0 N) for 4 hours in a closed container in the fume hood to eliminate all microorganisms such as mold, fungi, bacteria and viruses present on the seed surface. Next, seeds in 1 ml of sterile distilled water (dH2O) were incubated at 4 °C for a week to break seed dormancy, and then carefully planted on round plates containing ½ Murashige and Skoog (MS) media (Murashige & Skoog, 1962), 0.6% agar and 10 M gibberellic acid to trigger seed germination. These plates were placed horizontally at 20 °C under optimum light conditions (cool white fluorescent) in a plant growth chamber for at least two weeks. After each seedling was old enough for planting, the seedlings were carefully transferred to fully wetted soil and maintained under long-day conditions (16 h light and 8 h dark) at 24 °C in the greenhouse. Greenhouse grown plants were watered as needed to avoid water stress. Growth chamber and greenhouse conditions were carefully monitored to maintain healthy growth and development of plants.

2.2. Mutant Isolation

Mutant isolation was achieved by three steps: designing specific primers for genotyping, extracting DNA from plant tissues, and genotyping of each mutant line.

2.2.1. Primer design

Two types of molecular marker were used to genotype the plants. These markers are CAPS; a cleavage of amplified polymorphic sites, and dCAPS; derived CAPS. CAPS PCR primers have been first designed for detecting polymorphisms between two *Arabidopsis* ecotypes L*er* and Col (Bell & Ecker, 1994), and then for specific single point mutations (Neff et al., 2002). For all the TILLING alleles, the mutation is only a change of a single base pair in *DDL*. These single changes can be identified using the CAPS or the dCAPS markers. CAPS and dCAPS are based on the different pattern of digestion between the wild type and mutant alleles due to mutation altering an enzyme restriction site. dCAPS is a modification of CAPS technique that allows detection of most single nucleotide changes by utilizing mismatched PCR primers (Michaels & Amasino, 1998; Neff et al., 1998). The mismatches in the PCR primers, in combination with the single nucleotide change create a unique restriction site in one of the alleles. Polymorphism is revealed by the digestion of PCR product with a unique restriction enzyme, which will cut either the wild type or the mutant DNA. Agarose gel analysis of the restriction digestion pattern of PCR products determines the genotype of each plant. For PCR of TILLING lines, CAPS and dCAPS primer sets were designed using the web based program, dCAPS Finder 2.0 (Neff et al., 2002). Primers designed for each mutant line were listed (Table 2).

Table 2. Primer pair designed for TILLING mutant lines*

TILLING Lines	Marker	Primer Sequences Forward (F) Reverse (R)	Annealing Temp. (°C)	PCR Product (bp)
2182	dCAPS	F: GGTTTCCCATTATGAATCATACCCC R: TTCTCGTGCAACAGTACGTAAGCT	53	142
2206	dCAPS	F: GAATGTGCTTTTCTCAGGGAGAAG R: AAATTTAGCTTAAAGCCAGCAGA	52	130
2317	CAPS	F: GATCTGATATGTTTGTGACTGTGGC R: CCGTAGGAATGTCGGCAATCC	57	245
955	CAPS	F: AGGAAAGTCCCATTGAGCCAC R: TCACAGAGAATAGGCACGGGG	53	145
731	CAPS	F: CCCATTATGAATCATACCCCGTGCC R: TCGGCAGAATTCTCGTGCAACAGGA	57	145

**All primer sequences are written from 5' to 3'. Mismatched nucleotides introduced for the dCAPS primers are indicated in red. CAPS primers were designed to introduce point mutation in the PCR product spanning a restriction enzyme site. dCAPS primers were designed by generating mismatches to cleave only the wild type PCR product.*

2.2.2. DNA isolation

Modified Dellaporta plant DNA extraction protocol was used to isolate DNA from wild type and mutant plants (Dellaporta et al., 2007). A 0.5 cm^2 piece of fresh leaves from young seedling of mutant and wild type plants growing on soil were collected, and quickly frozen in liquid Nitrogen. They were then ground in a centrifuge tube using a pestle. 400 µl of Dellaporta buffer (100 mM Tris-HCl pH 8,50 mM EDTA, 500 mM NaCl, 350 µl β-Mercaptoethanol, and 20% SDS) was added to the leaf powder. The samples were incubated at 65 °C for 20 minutes in a water bath. During incubation, the contents were mixed a few times by inverting the tubes gently, and then 155 µl of Solution 3 (5 M potassium acetate, 5 M acetate, 57.5 ml glacial acetic acid and dH_2O) was added. Each sample was mixed well by vortexing, and then centrifuged at 13,000 g force for 10 minutes at room temperature. The supernatant was transferred to a new tube. To precipitate DNA, 500 µl of isopropanol was added, samples were mixed by inverting gently, and then incubated at -80 °C for 10 minutes. Each sample was centrifuged at 13,000 g force for 20 minutes at 4 °C, and the supernatant discarded. Samples were dried in speed vacuum and then dissolved in 100 µl sterile distilled water (dH_2O).

2.2.3. Genotyping: PCR- gel electrophoresis- restriction digestion

The Polymerase Chain Reaction (PCR) is a technique that is used to amplify DNA of interest at the detectable levels. After PCR, products are analyzed by gel electrophoresis to make sure that correct sized DNA fragments are generated (Mullis et al., 1992). The DNA isolated from mutant plants

with point mutation in *DDL* gene was used as template in PCR for amplification of the region of interest. As a positive control, L*er gl-1* genomic DNA was used. As a negative control, no genomic DNA was used for PCR reaction.

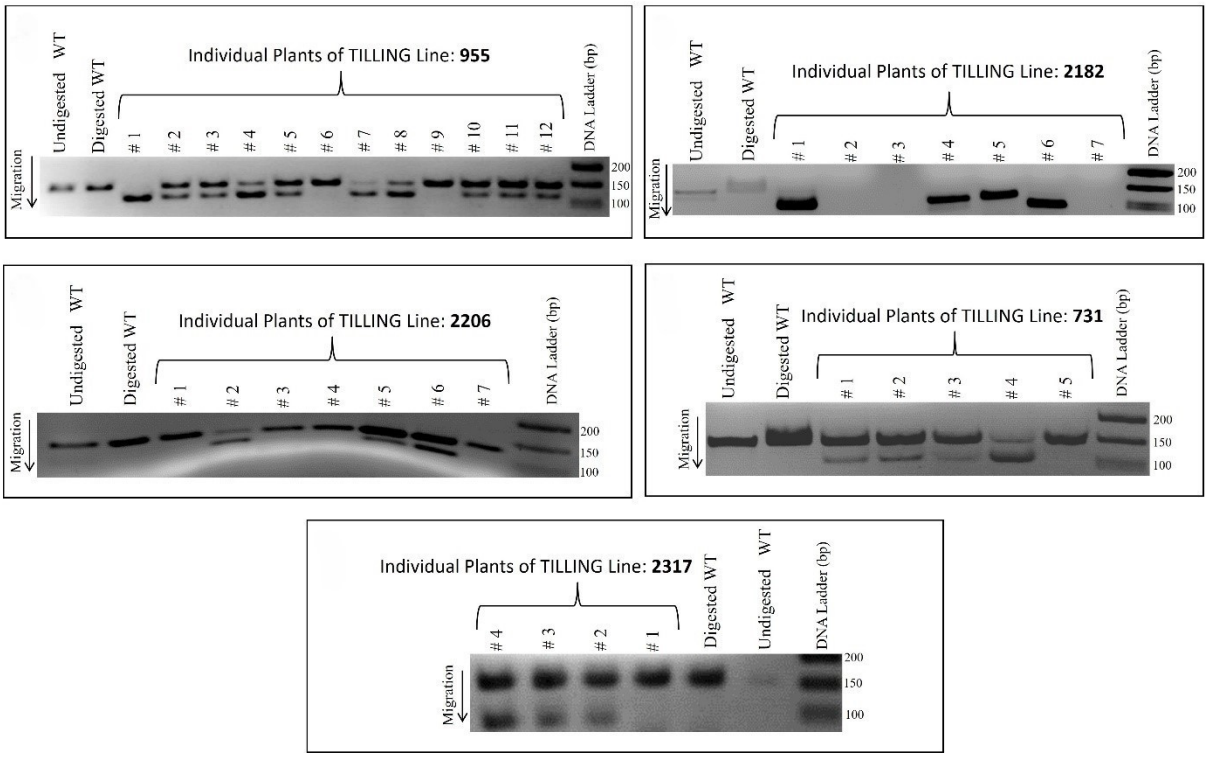

Figure 2. Isolation of homozygous individuals of TILLING mutant line

All PCR amplifications were performed in 30 µl of reaction volume containing 1.5 µl of template DNA, 1-unit of *Taq* polymerase, 3 µl of 10 mM dNTP, 0.5 µl of 20 mM primers for both forward and reverse CAPS and dCAPS primers, and 3 µl 10X reaction buffer. The amplification was carried out using a thermal cycler (Bio-Rad DNA Engine, USA). PCR cycles were programmed as 5 minutes for the first denaturation, then following 45 seconds for second denaturation step at 94 °C, 45 seconds for annealing step at 50–57 °C (depending on melting temperature of each primer pair used for mutant lines), and 30 seconds for elongation step at 72 °C. Each cycle from second denaturation to elongation step was repeated as 29 times. Finally, 5 minutes for extension cycle at 72 °C was put into PCR program to ensure the completion of amplification of all the target DNA templates. 10 µl of PCR products was used for initial gel electrophoresis to confirm the DNA region of interest was well amplified. The remaining PCR product (20 µl) was used for restriction digestion and gel electrophoresis. DNA samples were run on 1.5 % agarose gel (Bartlett, 2003), and visualized under ultra violet (UV) light (FOTODYNE Transilluminator, USA). After confirming that PCR worked, appropriate 1 unit of restriction enzyme for each mutant line (Table 3) and 2 µl of specific enzyme buffer were added to each 20 µl of PCR products, mixed well by inverting 2-3 times and then the samples incubated at 37 °C for overnight.

Table 3. Digestion patterns of each TILLING mutant line*

TILLING lines	Restriction enzyme	Restriction buffer	Recognition sequence
2182	HindIII	E	5'...A^AGCTT...3' 3'...TTCGA^A...5'
2206	MboII	NE4	5'...GAAGA(N)$_8$^...3' 3'...CTTCT(N)$_7$^...5'
2317	NlaIII	NE4	5'...CATG^...3' 3'...^GTAC...5'
955	Ssp1	N2	5'...AAT^ATT...3' 3'...TTA^TAA...5'
731	Fok1	E	5'...GGATG(N)$_9$^...3' 3'...CCTAC(N)$_{13}$^...5'

**The indicated restriction enzymes were used to cut genomic DNA amplified by PCR to identify mutant alleles of each TILLING line. (^ and ^; cleavage sites)*

Restricted PCR products were run on 2.8% agarose gel due to smaller DNA fragments cut by the restriction enzyme (Figure 2). This process was repeated until obtaining homozygous individuals for each mutant line.

In these agarose gel pictures; digested PCR products of TILLING mutant lines 955 (A), 2182 (B), 2206 (C), 731 (D), 2317 (E) and wild type plants were run on 2.8% w/v gel to isolate homozygous mutant individuals. Digested wild type PCR products were used as positive control. Homozygous individuals of the TILLING mutant lines are distinguished as a single cut lower DNA band, as shown in plant sample number #1 of the TILLING 2182 mutant line, while heterozygous individuals are recognized as two DNA bands containing cut lower and uncut upper DNA bands as shown

in plant samples number #2, #3 and #5 of the same mutant line. The expected sizes of cut and uncut DNA bands are confirmed by Mini DNA molecular weight marker represented in the last line on the right. Arrow shows the direction of migration of digested and undigested PCR products through the gel. (WT; wild type, bp; base pair).

2.3. Backcrossing

Because chemical mutagenesis creates background mutations, backcrossing to the wild type plant was performed to remove background mutations created by EMS. For backcrossing, pollen from wild type Ler gl-1 and pistils from homozygote TILLING lines were used. All young flower buds and flowers with petals of mutant plants were removed, and a few large buds were left. These buds were opened and six anthers were removed without damaging the pistil. This was performed under low magnification on a stereo microscope. Anthers with pollen seen as yellow powder from fresh fully open flowers of wild type plants were taken, and then the anthers were brushed to the stigma of mutant plant. Backcrossing steps were done several times between wild type and for each homozygous mutant plant. After three or four days, the pistil develops as a young silique if the backcrossing was successful. Seeds from backcrossing were named F1, and harvested when the siliques were brown and not dehisced yet after two or three weeks.

2.4. Phenotypic Characterization of TILLING Mutant Lines

Because ddl mutant plants displayed pleiotropic phenotype such as shortened and delayed vegetative organs and reduced fertility, it is essential to characterize the TILLING mutants after backcrossing. Homozygous individuals isolated and genotyped from each TILLING line were characterized for root and hypocotyl length, seed number and plant height to identify whether single nucleotide polymorphic alleles of DDL among the TILLING mutant lines have an effect on phenotype.

2.4.1. Root and hypocotyl length

To measure root and hypocotyls length of mutant and wild type plants, seeds of those plants were sterilized, cold treated, and plated on square plates with ½ MS agar with 10 M GA (gibberellic acid). After red light treatment of 1 hour following growth in the dark of 23 hours to trigger simultaneous germination of all seeds, the plates with seeds were put in growth chamber vertically. After 9 days of growth, the seedlings of each mutant line and wild type from the same plate were transferred to a transparency sheet. Hypocotyls and root lengths of plants were marked using a black marker, and then these sheets were scanned for measurement. Scanned picture of each plate was edited using Adobe Photoshop Elements 2.0 program (Adobe Systems Incorporated, USA), and the lengths were measured using Image J program (The Research Services Branch of the National Institute of Mental Health, USA). After getting average lengths of hypocotyls and root of mutant and wild type plants, data were analyzed statistically to identify whether there is a significant difference between wild and mutant lines in the length of root and hypocotyls by performing SAS (Statistical Analysis System) program (SAS Institute, USA).

2.4.2. Seed number and plant height

For counting seed number and measuring of heights of mutant and wild type plants, sterilization, cold treatment, plating of the seeds, and transferring the seedlings from the MS plates with GA to the soil were done as mentioned in plant material and growth condition section. After 45 days of growth, the heights of mutant and wild type plants were determined by measuring the distance between soil level and apical (terminal) bud. For seed number, total 30 siliques from different individuals of each line were dissected carefully and magnified under light microscope. The seeds into each silique were counted. The results of measurements of plant height and seed number were analyzed in SAS program to determine whether there was a significant difference between mutant and wild type plants in terms of seed number and plant heights.

3. RESULTS

3.1. Isolation of New ddl Alleles

To identify whether FHA domain and C-terminal region of DDL protein are required for its function, the seeds from five different TILLING mutant lines having a single nucleotide mutation created by EMS were ordered. A homozygote mutant for all of the five TILLING lines was isolated.

3.2. Backcross of the New ddl Alleles

One backcross has been successfully performed for the following TILLING lines: 2182, 2206 and 2317. However, TILLING lines; 955 and 731, did not give any heterozygous F_1 progeny seed from the backcrossing with wild type L*er gl-1*.

3.3. Phenotypic Characterization of the New ddl Allele

Since TILLING lines 955 and 731 did not give any F_1 progeny seed from the backcross, TILLING lines; 2182, 2206 and 2317 homozygous plants have been characterized at the phenotypic level after the first backcrossing. F_3 plants of the first backcross were used for phenotypic analysis.

3.4. Root and Hypocotyl Length

9 days old plants from *ddl-1* null mutant and Ws-2 wild type were used as positive control for analysis. *ddl-1* mutant plants showed an essential difference in the length of root and hypocotyls compared to Ws-2 wild type. Similarly, 9 days old plants from isolated TILLING mutant lines; 2182, 2206 and 2317 displayed shorter root (Figure 3) and hypocotyl (Figure 4) lengths having statistical importance at α-value 0.05, compared to L*er gl-1* wild type plants under the same growth and environmental conditions. (When the p-value of the statistical test, the probability of obtaining a test statistic, is less than α-value 0.05, the result is said to be statistically significant.

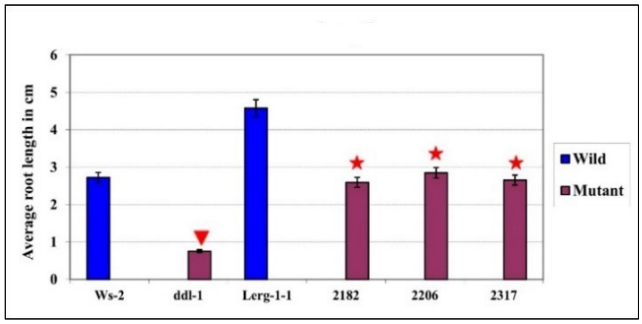

Figure 3. The average root length of TILLING mutant lines

(The 'x' axis indicates the genotypes used for root analysis. The 'y' axis represents the average root lengths in cm. The triangle denotes a statistical significance of decreased root length of ddl-1 null mutant plants compared to Ws-2 wild type plants at α-value 0.05. Similarly, the star denotes a statistical significance in the reduced root lengths of each TILLING mutant line compared to Ler gl-1 wild type plants. The sample size was 21 plants for each genotype. ddl-1 null mutant and Ws-2 wild type were used as positive controls for analysis)

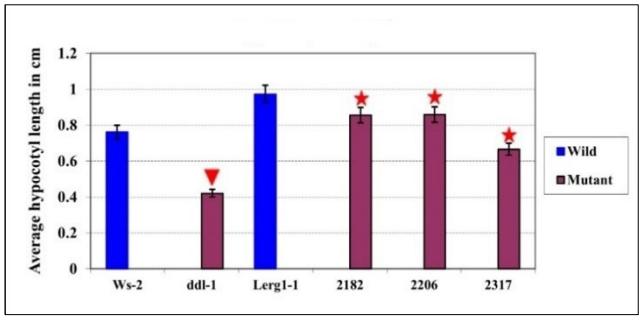

Figure 4. The average hypocotyl length of TILLING mutant lines

(The 'x' axis represents the genotypes used for hypocotyls analysis. The 'y' axis indicates the average hypocotyl length in cm. The triangle on the chart indicates a statistical significance of decreased hypocotyl length of ddl-1 null mutant plants compared to Ws-2 wild type plants at α-value 0.05. Similarly, the star denotes a statistical significance in the reduced hypocotyls lengths of each TILLING mutant line compared to wild type Ler gl-1 plants. The sample size was 21 plants for each genotype. ddl-1 null mutant and Ws-2 wild type were used as positive controls for analysis)

3.5. Plant Height and Seed Number

45 days old plants from *ddl-1* null mutant and Ws-2 wild type were used as positive controls for the analysis. *ddl-1* mutant plants showed a significant difference in the height of plant and seed number per silique compared to Ws-2 wild type. Similarly, 45 days old plants from isolatedTILLING mutant lines; 2182, 2206 and 2317 displayed statistically significant shortening in plant height (Figure 5) and reduction in seed number per silique (Figure 6) with a α-value of 0.05 when compared to wild type plants under the same growth and environmental conditions.

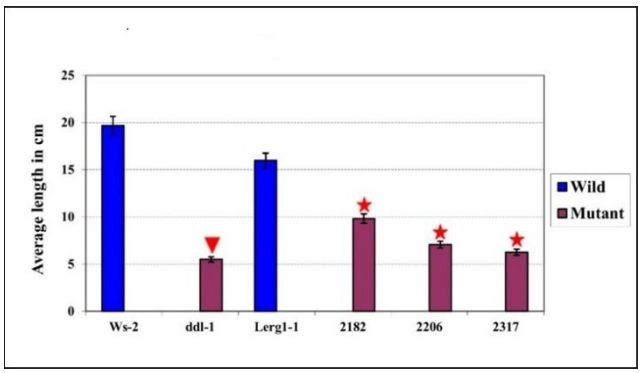

Figure 5. The average height of plants of TILLING mutant lines

(The 'x' axis indicates the genotypes used for the analysis of plant height. The 'y' axis represents the average plant height in cm. The triangle on the chart indicates a statistical significance of shortened plant height of ddl-1 null mutant plants compared to Ws-2 wild type plants with an α-value of 0.05. Similarly, the star indicates a statistical significance in the decreased plant heights of each TILLING mutant line compared to wild type Ler gl-1 plants. The sample size was 21 plants for each genotype. ddl-1 null mutant and Ws-2 wild type were used as positive controls for analysis)

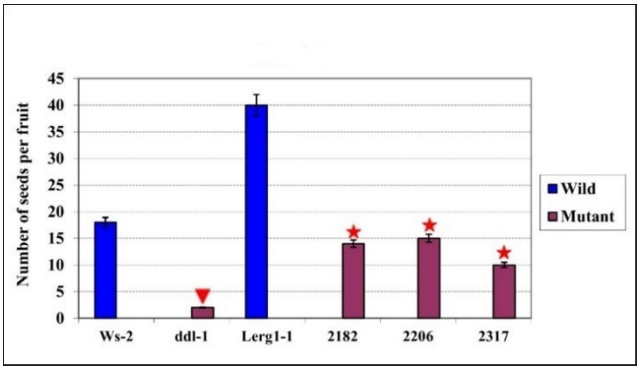

Figure 6. The average seed number of TILLING mutant lines

(The 'x' axis indicates the genotypes used for determining the number of seeds per silique. The 'y' axis represents the average number of seeds present in a silique. The triangle on the chart indicates a statistical significance of reduced seed number of ddl-1 null mutant plants compared to Ws-2 wild type plants with an α-value of 0.05. Similarly, the star denotes a statistical significance in the decreased seed number of each TILLING mutant line compared to Ler gl-1 wild type plants. The sample size was 30 plants for each genotype. ddl-1 null mutant and Ws-2 wild type were used as positive controls for analysis)

4. DISCUSSION

In this study, structure-function analysis of EMS-induced point mutations in the FHA domain and C-terminal region of the *DDL* gene was performed. 5 different TILLING point mutants (TILLING 2317, 2206, 955, 2182 and 731) were isolated, but 3 of it (TILLING 2206, 2317 and 2182) were characterized at phenotypic level. For each mutant line, homozygous individuals were isolated and backcrossed with wild type L*er gl-1*. However, TIILLING mutant lines 955 and 731 did not produce any seed when crossed to wild type L*er gl-1*, and therefore have not been characterized to date. One possible reason for the failure to obtain F_1 seed may be that physical and physiological conditions caused by genomic differences reduce the probability and success of fertilization. In previous study, F_1 seed production from crossing of different ecotypes of *Arabidopsis* has not been fully successful due to ecotype differences and therefore incompatibility of possible different gene regions in the genome that could be effective (Burkart-Waco et al., 2011). In addition, reproduction in flowering plants depends on the interaction and communication processes between pollen and floral organs. Failures in this interaction necessarily impair fertility, embryo and seed formation. In

Arabidopsis, a mutant called feronia has been isolated that showed a disruption for this interaction (Huck et al., 2003). This mutant demonstrated the possibility that mutating of a gene or gene regions in the genome can interfere with the formation of the F_1 seed from a cross with parental plants. Another possible reason may be the presence of other background mutations created through EMS mutagenesis that may negatively affect the structure of floral organs of TILLING mutant plants, preventing fertilization or if fertilization takes place, there may be no growth and development of F_1 seed from backcrossing. However, TILLING 2182, 2206, and 2317 mutant lines successfully produced heterozygous F_1 progeny seeds.

Phenotypic analyses of the TILLING 2182, 2206 and 2317 mutants revealed significant effects, resulting in reduced root and hypocotyl length, shortened plant height, and decreased seed number per silique compared to positive controls. This effect can be attributed to the fact that a change in the amino acid sequence of a protein due to a point mutation can lead to misfolding, structural deformation and consequently alter the activity of the protein (Matthews, 1987; Pandey et al., 2023). Therefore, a structural change in the amino acid sequence can cause striking or pleiotropic differences at the phenotype level, which have already been demonstrated in the analysis of *DDL* point mutations (Narayanan et al., 2014). A significant decrease in root and hypocotyl length, a remarkable reduction in plant height and seed number were two phenotypes which *ddl* mutants consistently displayed. TILLING 2182, 2206 and 2317 point mutants showed similar phenotypes to two T-DNA insertion mutant lines, *ddl-1* and *ddl-2*, (Morris et al., 2006) and TILLING point mutants in the FHA domain, *ddl-3*, *ddl-4* and *ddl-5* (Narayanan et al., 2014). The most dramatic phenotype in all TILLING mutants was observed in seed number. All mutations resulted in significantly reduced seed number as reported previously (Narayanan et al., 2014), These data may suggest that the FHA domain and C-terminal region are essential for function of DDL protein. However, additional data is required to confirm this conclusion and to elucidate correlation between mutant phenotypes and *DDL* function. For example, a second and further backcrosses between mutant lines and wild type background should be carried out to decrease the possibility of potential functional effect of any other background mutations created by EMS in different region of the genome. It is a well-documented phenomenon that repeated backcrossing results in a 50% reduction in heterozygosity in each subsequent generation. This implies that in the F_1 generation following the second backcross, approximately 50% of the progeny will exhibit heterozygosity, while the remaining 50% will be homozygous for the wild type (Karunarathna et al., 2021). This can reveal whether point mutations have a direct impact on *DDL* function. In addition, mRNA levels of *DDL* of each mutant line must be measured by RT-PCR to verify whether those mutant lines have altered *DDL* mRNA transcript levels, and to definitely prove DDL protein is still present in TILLING mutant lines. It is expected that mRNA levels of *DDL* is decreased due to point mutations as reported by previous studies (Morris et al., 2006; Narayanan et al., 2014). Furthermore, because DDL functions in biogenesis of miRNAs, and *ddl-1* mutant has reduced amount of pri and pre-miRNAs (Yu et al., 2008), transcripts levels of miRNAs in TILLING mutant lines must be measured to assess the point mutation effect at the molecular level, and to elucidate more precisely the role of FHA domain and C-terminal region in DDL function. Future experiments that characterize or identify changes in gene expression patterns in *ddl* mutants will also help uncover the role of *DDL* in plant growth and development. Further identification and characterization of key residues in the FHA domain and C-terminal region will serve as an important tool for elucidating the DDL signalling pathway, as well.

5. CONCLUSION

To clarify whether the FHA domain and C-terminal region are necessary for DDL function, five different TILLING mutant lines were isolated, and three of them (2182, 2206 and 2317) were analyzed at the phenotypic level. After the identification of homozygote mutant following the first backcross, the phenotype of F_3 plants of the first backcross was quantified based on the displayed phenotypes of *ddl-1* knockout mutant, and wild type Ws-2 and L*er gl-1* which were used as a positive control. Phenotypic analysis involved measuring the height of the plants on soil, the length of root and hypocotyl on vertical plates, and fertility assessment by counting the number of seeds per silique. TILLING mutant lines 2182, 2206 and 2317 displayed statistically significant shortness in plant height and the length of root and hypocotyl, and a reduction in seed number per silique as *ddl-1* null mutant does. Isolated and phenotypically characterized these TILLING mutant lines carrying a single nucleotide change created by EMS mutagenesis in different exon regions coding FHA domain and C-terminal region of DDL protein partially elucidates whether FHA domain and C-terminal region are required for DDL function in plant growth and development.

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Author Contribution

Seyit YUZUAK: (b) Methodology, (c) Literature Review, (f) Data collection and Processing, (g) Formal analysis, (h) Writing- Original draft preparation.

David CHEVALIER: (a) Idea and Concept, (b) Study Design and Methodology, (d) Supervision, (e) Material and

Resource Supply, (g) Analysis, (i) Critical Reviewing and Editing

Declaration of Ethical Code

The authors declare that this study is an original study; conducted in accordance with the principles and rules of scientific ethics at all stages of the study, including preparation, data collection, analysis and presentation of information; cited sources for all data and information and included these sources in the bibliography; have not made any changes in the data used. The authors comply with ethical duties and responsibilities by accepting all the terms and conditions of the publication ethics within the scope of the "Directive on Scientific Research and Publication Ethics of Higher Education Institutions" and also declare that none of the actions specified under the heading "Actions Contrary to Scientific Research and Publication Ethics" of the aforementioned directive have been carried out. The authors hereby declare that if at any time a situation contrary to this statement made regarding the study is detected, they agree to all moral and legal consequences that may arise.

Conflict of Interest

The authors report there are no competing interests to declare.

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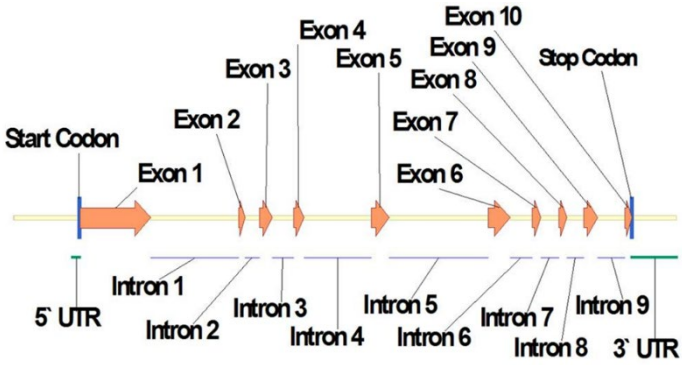
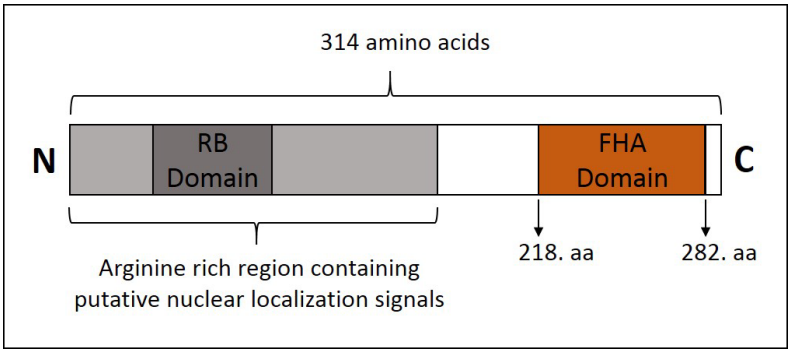
SUPPLEMENTARIES

Supplementary Figure 1. Nucleotide sequence of the complement strand of At3G20550 (*DDL*) gene located on Chromosome 3 (TAIR SeqViewer, 2021)



Supplementary Figure 2. Genomic structure of the *DDL* gene (3005 bp from Start to Stop codon). (*Orange arrows represent coding sequences (exons). Purple lines represent noncoding sequences (introns). The 5' and 3' untranslated regions are shown in green lines*)



Supplementary Figure 3. Domains in the DDL protein. Amino acid residues 218 to 282 towards the C terminus of the protein form the FHA domain of DDL. *(RB: RNA-Binding, FHA: Fork-Head Associated, aa: amino acid, C: C-terminal, N: N-terminal)*