Available online: April 22, 2019

Commun.Fac.Sci.Univ.Ank.Series C
Volume 28, Number 1, Pages 43-66 (2019)
ISSN 1303-6025 E-ISSN 2651-3749
https://dergipark.org.tr/tr/pub/communc/issue/45050/562280

COMMON BEAN (*Phaseolus vulgaris* L.) *DOF* TRANSCRIPTION FACTORS DIFFERENTIALLY EXPRESSED UNDER SALT STRESS

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ABSTRACT. DNA-binding with one finger (*DOF*) transcription factor family has found to be playing important roles for seed germination, photosynthesis, plant development and stress responses in several plant species. Hence, this study aimed to characterize the DOF genes in common bean at the genome-scale level. The various bioinformatics tools were used and the results were confirmed through bench-work studies. Expression levels of putative *PvDOF* genes were analyzed using publicly available RNA-seq data and the expression levels of five selected *PvDOF* genes were further analyzed through qPCR in tolerant cv. 'Yakutiye' and sensitive cv. 'Zulbiye' subjected to salt stress. As a result, 42 candidate *PvDOF* genes were defined. The length of PvDOF proteins ranged from 181 (PvDOF-41) to 503 (*PvDOF-35*) amino acids (aa). pIs of *PvDOF* proteins were between 5.03 (*PvDOF-21*) and 8.92 (*PvDOF-6*) ranging from acidic to alkaline, and the molecular weight of *PvDOFs* were between 21944.3 Da (*PvDOF-14*) and 54786.5 Da (*PvDOF-35*). While the highest number of *PvDOFs* was found on chromosome 2 (eight genes), the lowest number of *PvDOF* genes was identified on chromosomes 4 and 7 (one gene). Two segmentally duplicated gene couple were detected. A total of 21 *PvDOF* genes were targeted by miRNAs of 20 plant species. According to the normalized RPKM (Read Per Kilobase Million) values which were obtained from the RNAseq analysis, *PvDOF-6, 8, 9, 17, 27, 28, 30, 35* and *39* genes were found up- or down-regulated after salt stress treatment in the leaf and root tissues of common bean. Additionally, the most of the qPCR data were found to be consistent with the RNAseq data and the *PvDOF17* gene was found as being the most expressional divergent gene between cv. 'Zulbiye and cv. 'Yakutiye'. In conclusion, the results of this study might help understanding the biological roles of *PvDOF TF* family under salt stress and can be used for the improving of common bean through biotechnological approaches.

Received by the editors: March 13, 2019; Accepted: March 30, 2019.
Key word and phrases: Bioinformatics, *DOF*, RNAseq, qPCR, *Phaseolus vulgaris*, salinity

1. Introduction

Phaseolus vulgaris L. is the most important vegetable crop for human nutrition worldwide, which provides protein, vitamins and minerals for human diets [1, 2]. Total world production is approximately 21 million metric tons (MT) per year and this production amount is more than half of the world's total legume production. In developing countries, bean production takes place in small farms under low input agriculture and the beans of these farms tend to be affected more by the biotic and abiotic stress conditions [2]. Hence to combat these stress conditions, these farmers also use pesticides, fertilizers and irrigation in a way, which seriously threaten the environment. Due to these reasons, researches which aim to develop resistant common bean cultivars against biotic and abiotic stresses are becoming increasingly important. It is thought that the use of cultivars with improved stress resistance can lead to more stable bean production across adverse environmental stress conditions

Regulation of transcription has an important role in gene expression and can be controlled by the transcription factors (TFs), which bind to the specific gene promoter sequences [3, 4, 5, 6, 7]. The DOF family is an important example of such transcription factors, which is a plant-specific transcription factor family containing a highly conserved DNA binding domain (Dof domain) [8]. The *DOF* proteins were found to have only one copy of the Dof domain in the N-terminal region and the rest of the amino acid sequences are very divergent [9]. Following the discovery of the first *DOF* protein (*ZmDof 1*) in maize, many other members of *DOF* proteins have been identified in various plant species up to date. These studies have demonstrated that *DOF TFs* play an important role in plant growth, development, seed germination, photosynthesis and stress responses in several plant species [10]. It was found that when plants are exposed to the diverse abiotic stress types, thousands of TFs, including *DOF* family genes, are activated to adjust the physiological processes and biochemical pathways [11, 12]. In this study, 42 *PvDOF* members were identified in common bean (*P. vulgaris*) and detailed analyses of the sequence phylogeny, genomic organization, gene structure, conserved protein motifs, gene duplication events, cis-elements and gene ontology (GO) were performed. Expression levels of putative *PvDOF* genes were also analyzed using publicly available RNA-seq data and further, the expression levels of five selected *PvDOF* genes were investigated using Quantitative Real-time PCR (qPCR) in two different common bean cultivars; cv. 'Yakutiye' and cv. 'Zulbiye'.

2. Material And Methods

2.1. Identification of *DOF* proteins in *Phaseolus vulgaris* genome

Sequences of the *DOF* gene family of *P. vulgaris* were obtained from Phytozome database v11 (www.phytozome.net) using key searching with Pfam Accession Number (PF04690), downloaded from Pfam Database (http://pfam.xfam.org/). To identify all the putative *DOF* proteins in *P. vulgaris*, both blastp at Phytozome database v11 (www.phytozome.net) and hidden Markov model (HMM), (http://www.ebi.ac.uk) searches were performed against the *P. vulgaris* genome [13]. Putative *P. vulgaris DOF* proteins were used for query in blastp (NCBI) for characterization of hypothetical proteins. Redundant sequences were removed by using the decrease redundancy tool (http://web.expasy.org/decrease_redundancy/) and sequences were checked for DOF domains by using HMMER (http://www.ebi.ac.uk) and Pfam databases. The physicochemical properties of DOF proteins were calculated by using ProtParam Tool (http://web.expasy.org/protparam).

2.2. Structure and physical locations of *DOF* genes, detection of gene duplication events and conserved motifs

The Gene Structure Display Server v2.0 (GSDS, http://gsds.cbi.pku.edu.cn/) was used [14] to obtain information on the exon – intron of *PvDOF* proteins. Both genome sequences and the coding sequences were utilized for predicting the positional information of the *PvDOF* genes by using Phytozome database v11, chromosomal locations. The sizes (bp) and intron numbers of *PvDOF* genes were identified. The *PvDOF* genes were plotted on all *P. vulgaris* chromosomes and pictured with MapChart [15]. Gene duplication events were detected through the alignment of the coding nucleotide sequences covered 70% of the longest genes and the amino acid identity between the sequences was >70% [16]. To identify additional conserved motifs of *PvDOF* proteins, we used the Multiple EM for Motif Elicition tool (MEME v4.11.1; http://meme-suite.org/) [17].

2.3. Phylogenetic analysis and sequence alignment

The multiple sequence alignment of *PvDOF* proteins was carried out using the ClustalW [18]. The neighbor-joining (NJ) method with bootstrap value of 1000 replicates (MEGA6) was used for constructing phylogenetic trees and the tree was designed using Interactive tree of life (iTOL; http://itol.embl.de/index.shtml) [19].

2.4. Subcellular Localization and Promoter Analysis of the *PvDOF* Family

The 5′ upstream regions (2 kb DNA sequence of each gene of the *PvDOF* family) were obtained from Phytozome database v11 (Supplementary Table S1) and analyzed using the plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for a cis-element scan. Subcellular Localization of the *PvDOF* proteins was determined using the WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html) [20] and TargetP 1.1 (http://www.cbs.dtu.dk/services/ TargetP/) [21].

2.5. In silico prediction of miRNA targets in *PvDOF* genes

All known plant miRNA sequences were obtained from miRBase v21.0 (http://www.mirbase.org). psRNA Target Server (http://plantgrn.noble.org/psRNATarget) with default parameters was used for prediction of miRNAs accordingly [22]. In-silico predicted miRNA targets were identified by BLASTX with ≤1e-10 against common bean Expressed Sequenced Tags (ESTs) at NCBI database.

2.6. Prediction of Synonymous and Non-Synonymous Substitution Rates

CLUSTALW software was used for the prediction of amino-acid sequences of tandemly and segmentally duplicated *PvDOF* genes. The CODEML program in PAML (PAL2NAL) (http://www.bork. embl.de/pal2nal) was used to estimate the synonymous (Ks) and non-synonymous (Ka) substitution rates [23]. Time (million years ago, Mya) of duplication and divergence of each *PvDOF* genes were determined using the following formula: $T= Ks/2\lambda$ ($\lambda = 6.56E-9$) [24, 25].

2.7. Identified Expression Level of *PvDOF* genes Through Transcriptome data

Illumina RNA-seq data was obtained from Sequence Read Archive (SRA) in order to measure the expression levels of *PvDOF* genes. The accession numbers SRR957667 (control leaf), SRR958472 (salt-treated root), SRR958469 (control root), and SRR957668 (salt-treated leaf) were used for this purpose [26]. The analysis of transcriptomic data was performed as described by Buyuk et al. (2016) [4]. Finally, the heat maps of hierarchical clustering were constructed using the PermutMatrix [27].

2.8. Plant materials, growth conditions, and stress treatments

The seeds of salt tolerant bean cv. 'Yakutiye' and salt sensitive bean cv. 'Zulbiye' were supplied from 'Transitional Zone Agricultural Research Institute, Eskişehir, Turkey' [28]. A preliminary experiment was performed to determine salt tolerance capacity of both common bean cultivars under severe salt stress conditions (250 mM and 400 mM NaCl for 9 days) by using malondialdeyhde (MDA) analysis in leaf tissues as an indicator test of stress. According to these preliminary data, salt tolerant cv. 'Yakutiye' seemed to be more resistant to salt stress than cv. 'Zulbiye' exhibited (Supplementary Figure S2).

For gene expression analysis, seeds of both cultivars were germinated, following the surface sterilization in a solution containing 5% (v/v) hypochlorite for 5 min, and were grown hydroponically in pots containing 0.2L of modified 1/10 Hoagland's solution. Hoagland solution includes macronutrients (K_2SO_4, KH_2PO_4, $MgSO_4.7H_2O$, Ca $(NO_3)_2.4H_2O$ and KCl) and micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4.5H_2O$, NH_4Mo, $ZnSO_4.7H_2O$) with a final concentration of ions as 2mM Ca, 10^{-6} M Mn, 4mM NO_3, 2.10^{-7} M Cu, 1mM Mg, 10^{-8} M NH_4, 2mM K, 10^{-6} M Zn, 0.2 mM P, 10^{-4} M Fe and 10^{-6} M B [29]. Common bean seedlings were incubated in a controlled environmental growth chamber in the light with 250 mmol m^{-2} s^{-1} photosynthetic photon flux at 25 ℃ and 70% relative humidity. Salt stress was applied with Hoaglands solution including 150 mM NaCl (for moderate salinity stress) for 9 days after common bean seedlings reached the first trifoliate stage in growth chamber. Following the ninth day of stress application, root and leaf tissues of two different common bean cultivars were sampled to be used in qPCR analysis. Three biological replicates were used for the qPCR reactions.

TABLE 1. The information of 42 PvDOF proteins.

ID	Phytozome Identifier	Chromosome Location	Molecular Weight (Da)	Isoelectric Point (pI)	Protein Length	NCBI Accession No.
PvDOF-1	Phvul.011G064800.1	Chr011:5629789…5631847	33520.4	9.53	309	XM_007132011.1
PvDOF-2	Phvul.008G099400.1	Chr008:10664656…10666281	37644.6	9.08	361	XM_007140225.1
PvDOF-3	Phvul.008G012500.1	Chr008:1111164…1112976	37827.3	9.31	350	XM_007139173.1
PvDOF-4	Phvul.010G013500.1	Chr010:2139058…2140753	35595.6	9.18	333	XM_007133969.1
PvDOF-5	Phvul.005G143100.1	Chr005:37185922…37187662	33380.3	9.58	305	XM_007150252.1
PvDOF-6	Phvul.002G022000.1	Chr002:2381355…2382917	35873.8	8.92	339	XM_007156780.1
PvDOF-7	Phvul.003G247900.1	Chr003:47381617…47383283	36064.1	8.64	338	XM_007155909.1
PvDOF-8	Phvul.009G204000.1	Chr009:30151578…30153734	34500.4	6.49	318	XM_007138322.1
PvDOF-9	Phvul.006G184000.1	Chr006:29308624…29310821	37332.6	8.68	345	XM_007148083.1
PvDOF-10	Phvul.003G182100.1	Chr003:39382825…39384177	30942.1	6.52	287	XM_007155140.1
PvDOF-11	Phvul.002G144900.1	Chr002:28149833…28151588	37051.7	8.81	344	XM_007158284.1
PvDOF-12	Phvul.003G287600.1	Chr003:51349107…51350494	37122.3	8.89	345	XM_007156395.1
PvDOF-13	Phvul.005G161200.1	Chr005:38639927…38641622	22040.6	9.02	205	XM_007150481.1
PvDOF-14	Phvul.010G141400.1	Chr010:41333505…41336027	21944.3	8.7	203	XM_007135519.1
PvDOF-	Phvul.002G230200	Chr002:39587482…3958	37110.0	9.06	347	XM_007159281.

15	0.1	9579				1
PvDOF-16	Phvul.005G13770 0.1	Chr005:36604141…36605891	31551.9	8.08	287	XM_007150172.1
PvDOF-17	Phvul.006G18410 0.1	Chr006:29322500…29324409	32201.4	9.26	297	XM_007148084.1
PvDOF-18	Phvul.007G26760 0.1	Chr007:50584990…50585817	30127.3	5.25	275	XM_007145724.1
PvDOF-19	Phvul.009G17830 0.1	Chr009:26082689…26084869	36775.1	6.58	336	XM_007138012.1
PvDOF-20	Phvul.003G20060 0.1	Chr003:41410491…41412765	35853.2	6.82	328	XM_007155369.1
PvDOF-21	Phvul.009G13640 0.1	Chr009:20017224…20018880	33109.5	5.03	298	XM_007137491.1
PvDOF-22	Phvul.011G07190 0.1	Chr0011:6441508…6443452	32876.5	7.51	298	XM_007132109.1
PvDOF-23	Phvul.001G19610 0.1	Chr001:46235911…46236696	29417.5	6.19	261	XM_007162914.1
PvDOF-24	Phvul.010G11560 0.1	Chr010:38220976…38223161	30649.0	9.04	282	XM_007135213.1
PvDOF-25	Phvul.006G11490 0.1	Chr006:23050676…23052519	27741.9	8.66	254	XM_007147270.1
PvDOF-26	Phvul.003G24850 0.1	Chr003:47458824…47459525	24497.0	8.72	233	XM_007155915.1
PvDOF-27	Phvul.001G06210 0.1	Chr001:7655746…7657228	29996.3	8.2	269	XM_007161292.1
PvDOF-28	Phvul.009G04750 0.1	Chr009:9121973…9124052	30490.0	8.88	278	XM_007136403.1
PvDOF-29	Phvul.002G22610 0.1	Chr002:39099335…39101145	34179.1	8.41	313	XM_007159235.1
PvDOF-30	Phvul.001G08080 0.1	Chr001:13241653…13243692	39112.8	9.22	355	XM_007161509.1
PvDOF-31	Phvul.002G23010 0.1	Chr002:39579558…39580512	27963.2	9.75	261	XM_007159280.1
PvDOF-	Phvul.006G18830	Chr006:29645412…2964	31113.5	8.12	280	XM_007148136.

32	0.1	7711				1
PvDOF-33	Phvul.002G238400.1	Chr002:40406454…40408578	20989.5	7.71	183	XM_007159386.1
PvDOF-34	Phvul.003G275800.1	Chr003:50194801…50197838	53028.3	8.03	480	XM_007156250.1
PvDOF-35	Phvul.008G037000.1	Chr008: 3099220…3102990	54786.5	6.55	503	XM_007139461.1
PvDOF-36	Phvul.002G084000.1	Chr002:13002774…13006753	52071.6	7.63	480	XM_007157549.1
PvDOF-37	Phvul.002G170600.1	Chr002:31594717…31597909	50325.7	7.57	464	XM_007158586.1
PvDOF-38	Phvul.009G186600.1	Chr009:27607268…27610475	52892.4	5.89	480	XM_007138113.1
PvDOF-39	Phvul.004G054000.1	Chr004: 6941656….6944670	49100.7	6.33	450	XM_007151459.1
PvDOF-40	Phvul.001G106400.1	Chr001:27221418…27224141	52006.5	7.43	475	XM_007161831.1
PvDOF-41	Phvul.006G176400.1	Chr006:28683988…28685016	20769.7	9.45	181	XM_007147989.1
PvDOF-42	Phvul.003G189300.1	Chr003:40146058…40144932	51448.8	5.2	472	XM_007155239.1

2.9. RNA isolation, cDNA synthesis and quantitative Real-Time PCR analysis

Total RNAs of leaf and root tissues of the two bean cultivars were extracted by using NucleoSpin® RNA Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. NanoDrop Lite UV–VIS spectrophotometer was used to determine RNAs quality/quantity and the presence of RNAs were also confirmed by gel electrophoresis which contains 1.5% agarose (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed with 2µg of RNA by using high fidelity cDNA synthesis kit (Roche), which contains 2.5µM Anchored-oligo $(dT)_{18}$, 1X transcriptor high fidelity reverse transcriptase reaction buffer, 20U protector RNAse inhibitor, 1mM deoxynucleotide mix, 5mM DTT and 10U transcriptor high fidelity reverse transcriptase at final concentration. The following incubation conditions were applied; 10 min at 65 ℃, 30 min at 55 ℃ and 5 min at

85 °C. cDNA quantity/quality was also measured with Nanodrop ND-Spectrophotometer Lite.

qPCR was performed with Light Cycler ® Nano System (Roche), thermal cycler. The primer sequences of the target genes and also the housekeeping gene, which is used for normalization, were designed with Primer3 program based on the sequences of 5 predicted *PvDOF*s (Supplementary Table S2). These 5 *PvDOF* genes were selected based on RPKM values obtained from expression analysis of *PvDOF* genes in RNA-Seq data. All qPCR reactions were performed in three independent biological and technical triplicates with a template free control to check any contaminations. Amplifications of PCR product were monitored with using an intercalator-based method including SYBR Green I dye. After predenaturation for 10 min at 95 °C, 45 cycles of 15 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C were applied. Melting-Curve analysis was performed to confirm the presence of a single product and absence of primer-dimers.

The abundance of target gene transcripts was normalized to ACT and set relative to control plants according to the $2^{-\Delta\Delta CT}$ method [30]. Changes in relative expression levels (REL) of the gene were checked for statistical significance according to one-way ANOVA. Fisher's least significant difference test at 0.05 significant levels was performed.

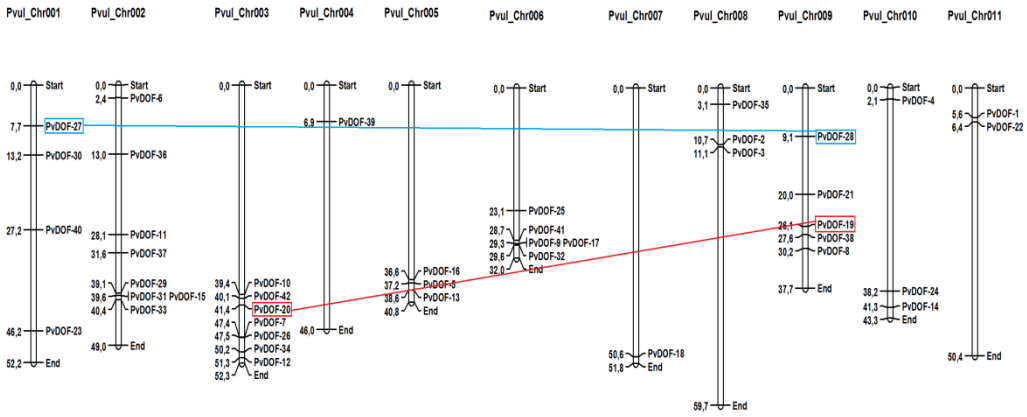

FIGURE 1. Distribution of *PvDOF* genes on common bean chromosomes.

3. Results And Discussion

3.1. Identification of *DOF* gene family in common bean

DOF proteins of the 15 different plant genomes were used as query sequences to identify the *DOF* genes in the *P. vulgaris* genome. Subsequently, DOF domains were searched by using HMMER and Pfam databases in putative PvDOF proteins and the redundant sequences were removed after the validation. We discovered 42 candidate *PvDOF* genes in common bean genome and listed them in Table 1 which involves the number of isoelectric point (pI), amino acids (length) and NCBI annotation. All of the non-redundant *PvDOF* genes were distributed on all chromosomes of common bean. While the lowest number of *PvDOF* genes was determined on chromosomes 4 and 7 (one *DOF* gene), the highest number of *PvDOF*s was found on chromosome 2 (8 *DOF* genes) (Figure 1). The length of *PvDOF* proteins ranged from 181 (*PvDOF-41*) to 503 (*PvDOF-35*) amino acids (aa). pIs of PvDOF proteins were between 5.03 (*PvDOF-21*) and 8.92 (*PvDOF-6*) ranging from acidic to alkaline, and the molecular weight of PvDOFs were between 21944.3 Da (*PvDOF-14*) and 54786.5 Da (*PvDOF-35*) (Table 1). To date, *DOF* family members in several plant species have been identified. Among the identified *DOF*s, the highest number of *DOF* genes was discovered in *Glycine max* genome which contains 78 *DOF* genes [31]. Furthermore, 76 genes in *Brassica rapa* L. ssp. *pekinensis* [10] , 52 genes in *Platycodon grandiflorum* A.DC. [32], 46 genes in *Daucus carota* L. subsp. *sativus* [33] , 42 genes in *Medicago truncatula* Gaertn. [34], 41 genes in *Populus trichocarpa* Torr. & A.Gray [35], 38 genes in *Cajanus cajan* (L) Millsp [36], 37 *DOF* genes in *Arabidopsis* [37], 36 genes in *Cucumis sativus* L. [38], 35 genes in *Solanum tuberosum* L. [39], 34 genes in *S. lycopersicum* L. [40], 33 genes in *Capsicum annum* L. [41], 30 genes in *Orzya sativa* L. [42] have been characterized. In this study, we identified 42 common bean specific *DOF* (*PvDOF*) genes distributed on all chromosomes.

Gene duplication is one of the most important characteristics of plant genomic structure, which can occur by independent mechanisms resulting in segmental or tandem duplications [43]. Due to the importance of gene duplications on evolution of gene families in plants, we have analyzed the gene duplication events of putative *PvDOF* genes in common bean genome. After the duplication analysis, we detected two segmentally duplicated gene couple (*PvDOF-19*/*PvDOF-20* and *PvDOF27/PvDOF28*) among the identified 42 *PvDOF* genes in common bean

(Supplementary Table S3). Ks/Ka distances and ratios were also estimated. The Ks of the segmental duplication of *PvDOF-19* and *PvDOF-20* was 0.7455, which dated the duplication event at 5.74 Mya; that the segmental duplication of *PvDOF-27* and *PvDOF-28* was 0.6997, dated the duplication event at 5.39 Mya (Supplementary Table S3).

In the previous studies, the duplication events in the *DOF* genes of Chinese cabbage [10], cucumber [36] and *Cajanus cajan* [38] were identified 10, 8 and 14, respectively. Intriguingly, we observed only two segmental duplication events in *PvDOF* genes. According to Gupta et al. (2015) [44], the divergence of monocots and dicots were observed 170-235 Mya. In this study, the mean date of segmental duplication events (5.565 Mya) showed that *PvDOF-19/PvDOF-20* and *PvDOF-27*/*PvDOF-28* genes were segmentally duplicated after the divergence of monocot-dicot split [38].

3.2. Phylogenetic analysis, conserved motifs, gene structure and subcellular localization of *PvDOFs*

To investigate the relationships among PvDOF proteins, a phylogenetic tree of *DOF* domain proteins in common bean, *Arabidopsis* and *Glycine max* was constructed using amino acid sequences with well-supported bootstrap values (1000 replicates) (Figure 2) through neighbor joining method. PvDOF proteins were classified into four groups (A to D), which could be further classified into 8 subgroups (Figure 2). Similar coding and exon-intron sequences were observed in most of the *PvDOF* genes, which were found in the same subgroup in phylogenetic tree.

To discover conserved motifs in PvDOF proteins, MEME (v4.11.1) was used. A total of 20 conserved motifs were identified (Supplementary Figure S2, Table S4). The lengths of discovered motifs ranged from 6 to 50 amino acids. All PvDOF proteins included Motif 1, which is the only motif that has DOF domain among all motifs. We investigated the gene structure of 42 common bean *DOF* genes. The predicted number of exons among the 42 *PvDOF* genes varied from one to three with 19 members having one and 21 with two (Figure 3). Two (*PvDOF-3* and *PvDOF-39*) had three exons. As seen, most of the *DOF* genes were found to include few introns (0-2) in common bean similar to that found in *Oryza* sp. and *Arabidopsis* previously [42].

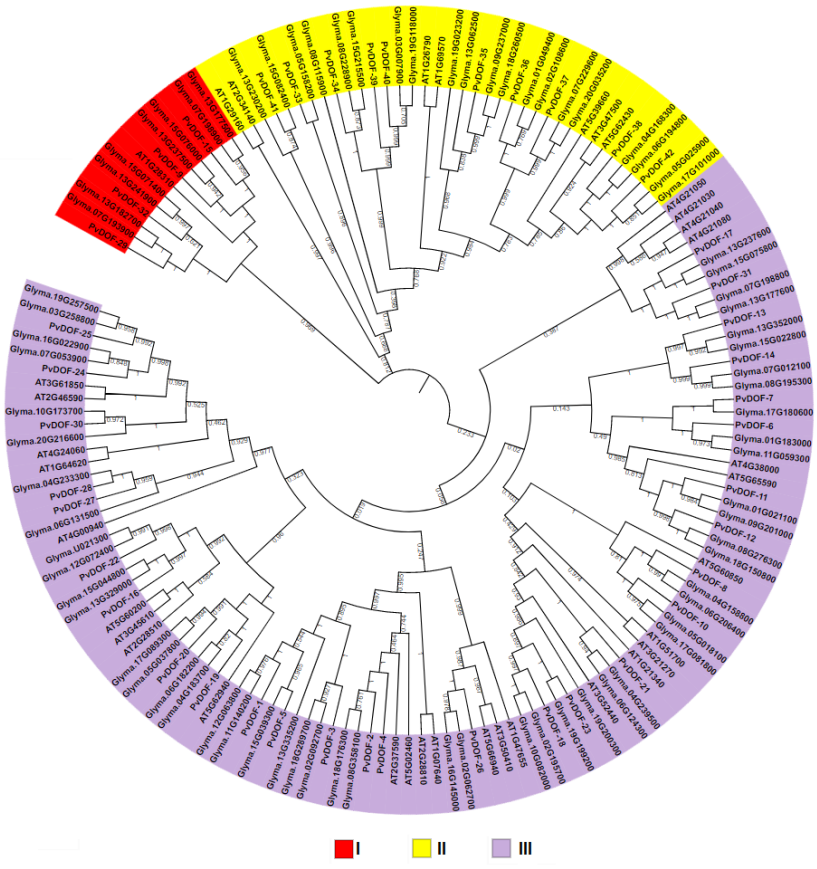

FIGURE 2. A phylogenetic tree of common bean *DOF* proteins

In addition, the number of introns within ORFs varied among *PvDOF* genes. 18 out of 42 genes did not contain any introns (Figure 3). Similar to our findings, 17 out of 36, 21 out of 43 [42] and 21 out of 28 [45] *DOF* genes were found intronless in rice, *Arabidopsis* and *Sorghum bicolor* (L.) Moench, respectively.

In this study, upstream promotor region of PvDOF family contained a total of 111 different cis-acting elements, which are categorized as light responsive elements

(41), development related elements (15), hormone responsive elements (14), environmental stress related elements (9), promoter related elements (6), site-binding related elements (4) and other elements (22) (Supplementary Table S5). Average number of cis-acting elements per *PvDOF* gene was 31.59 and the highest number (42) of cis-elements was found on *PvDOF-36* while the least (22) was observed on *PvDOF-34* and *PvDOF-39*. Recently, GT1 and TGACG motifs from a maize promoter were found to be related to salt stress tolerance [46] and these two cis-acting elements were also found in 27 and 21 of the *PvDOF* genes in common bean, respectively. In addition, predominance of many light responsive, development related, hormone responsive and stress responsive elements strongly indicates the involvement of *PvDOF* genes in abiotic and biotic stress response in common bean [36, 46].

In this study, all of the *PvDOF* genes were found to be located in nucleus according to the subcellular localization analysis. The TargetP analysis revealed that 8 PvDOF proteins were located in chloroplast and one was located in the secretory pathway (Supplementary Table S6).

3.3. Detection of miRNAs targeting *PvDOF* genes

According to the results of psRNATarget server, the maximum expectation and the accessibility of messenger RNA (mRNA) target site to miRNA ranged from 2.5 to 3.0 and 0.14 to 24.496, respectively (Supplementary Table S7). A total of 21 *PvDOF* genes (*PvDOF*-1, -2, -5, -6, -8, -13, -17, -19, -21, -22, -23, -24, -29, -30, -32, -33, -34, 36, -38, -39 and -41) were targeted by miRNAs of 20 plant species. Total of 38 miRNA families (miR156, miR167, miR319, miR395, miR403, miR407, miR414, miR482, miR779, miR854, miR859, miR1076, miR1311, miR1861, miR2082, miR3625, miR4241, miR4348, miR5035, miR5298, miR5565, miR5568, miR5658, miR6180, miR6253, miR6283, miR6287, miR7494, miR8121, miR8146, miR8181, miR8676, miR8722, miR9472, miR9494, miR9667, miR9723, miR9737) were detected. The most targeted genes were *PvDOF-8* and *-17*, which were targeted by five different plant miRNAs (Supplementary Table S7).

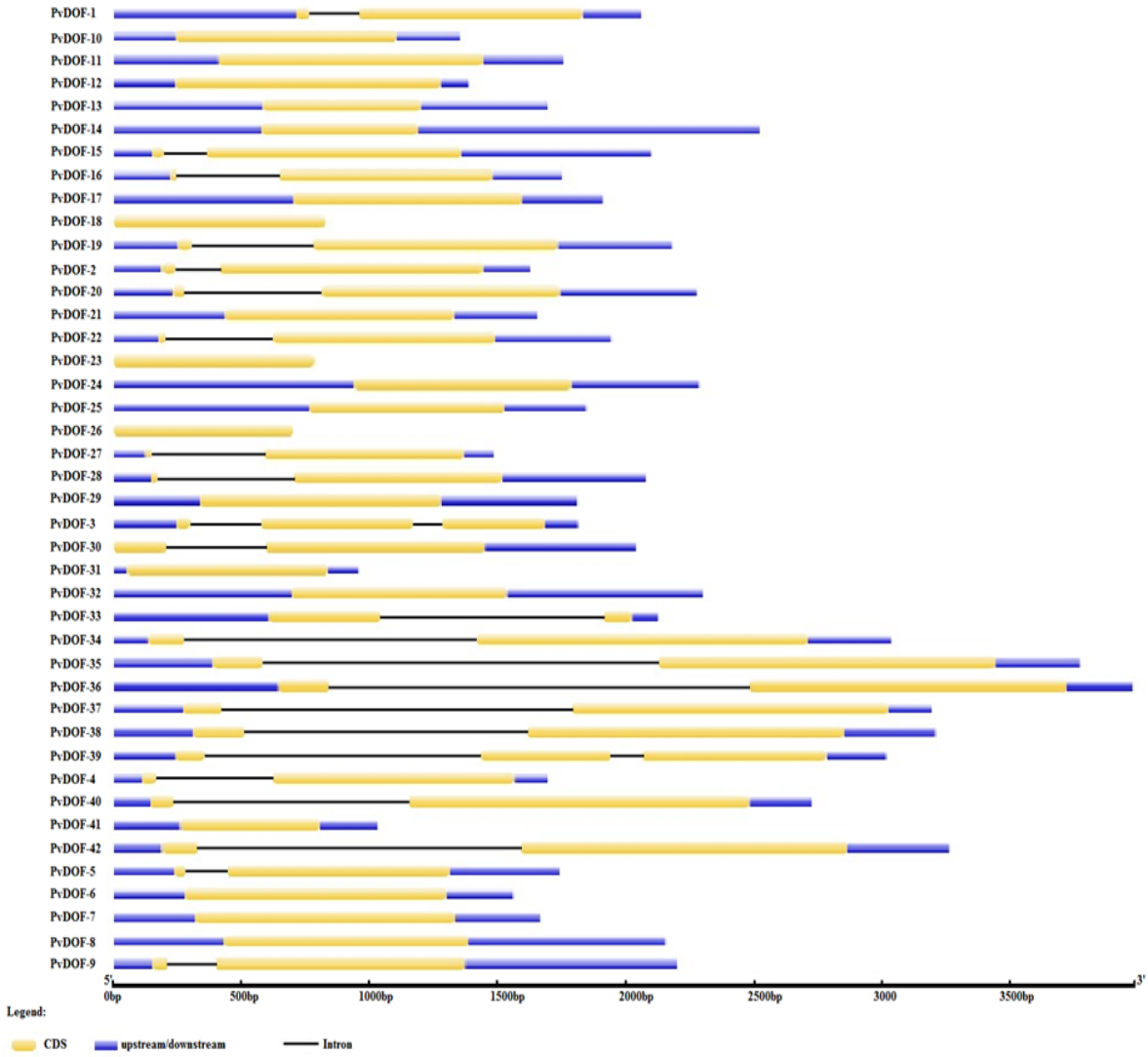

FIGURE 3. Gene structure of *DOF* genes.

Until today, miRNA-DOF connection was only found in the limited numbers of study. Song et al. (2016) reported that 13 *CmDOF*s genes were found to be targeted by 16 miRNA families [47]. They also validated that *CmDOF3*, 15, and 21 could be defined as the real targets of the plant miRNAs. Additionally, they found that

CmDOFs (3, 5, 6, 8, 9, 11, 15, 16, 18, 19, and 20) include only one target site. In this study, *PvDOF-5-8-19-21* were found to be targeted by miRNAs of 20 plant species. In another previous study, Wen et al. (2016a) [38] stated that, 15 *CsDOF* genes in cucumber genome were targeted by 20 miRNAs and *CsDof21* was found as being the most abundant transcript, which was targeted by six plant miRNAs (miR5021, miR5658, miR2673a, miR2673b, miR2673, and miR7494b). The sequence analysis of *CsDof26* showed that the miR319a targeted the region between 188–288 bp. Furthermore, miR5658 targeted *CsDof05, CsDof08, CsDof20* and *CsDof21* and *CsDof28* genes were also targeted by five different miRNAs (miR831, miR831-5p, miR393b-3p, miR393b-3p and miR3434-3p). In other previous transcriptome studies, miR156, miR167 and miR319 were found to be responsive to salinity stress in *Arabidopsis*, while miR854 and miR395 were found to be related with drought and copper stress, respectively [48, 49, 50]. In wheat, by using miRNA-microarray technology, expression level of miR482 was found to be important during the salt treatment [51]. Similar to those previously published studies, we found that all these stress related miRNAs target the *DOF* genes in common bean. These results might reflect the importance of these miRNAs and their target genes in the regulation of the *DOF* gene family in response to salt stress.

3.4. Genome Wide Expression Pattern of *PvDOF* genes

We used RNA-seq data sets, which were previously published by Hiz et al. (2014) for the identification of the DOF genes in common bean genome [26]. The expression profiles of the *PvDOF* genes were analyzed through that RNA-seq data, which had 52,858, 60,590, 51,564 and 59,510 unigenes in control leaf, control root, salt-treated leaf and salt-treated root, respectively. According to the normalized RPKM values, *PvDOF-6, -8, -9, -17, -27, -28, 30, -35* and *-39* genes were found to be up- or down-regulated in the leaf and root tissues of common bean under salt stress conditions (Figure 4).

In a previous study, *CsDof35* gene was found to be highly expressed under biotic stress conditions in cucumber genome [38]. Additionally, Song et al. (2016) [47] showed that the expression levels of the *DOF* genes were found as being variable in the different tissues of *Chrysanthemum morifolium* Ramat. Furthermore, they have proposed that the duplication events might be the reason of the divergence of *CmDOF6* gene expression level [47]. Accordingly, this study also revealed almost similar expression patterns with *CmDOF6* gene (Figure 4).

The expressions of *PvDOF-17, -28* and *-30* genes were up-regulated after salt stress treatment in leaves of common bean, while *PvDOF-8* and *-39* genes exhibited lower expression compared to untreated control sample. In addition, the expression level of *PvDOF-9, -27, -30* and *-35* genes were also found to be down-regulated in salt treated root samples, while *PvDOF-6 and -39* genes were found to be higher in salt treated root samples of common bean compared to untreated controls (Figure 4). *HvDof17* and *HvDof19* were found to have a role in the regulation of hormonal balance between gibberellin and abscisic acid during seed germination [52]. Associated with that result, abscisic acid was found to alter plant responses under salt stress [52].

3.5. Salt stress responses of *PvDOF* genes

In this study, we identified salt responsive *DOF* genes at transcriptome level in common bean genome. For this aim, five genes (*PvDOF-17*, *-27, -28, -30* and *-35*) were selected by taking the normalized RPKM values into consideration (Supplementary Table S2). Two different common bean cultivars which were previously classified as tolerant (cv.'Yakutiye') and sensitive (cv.'Zulbiye') were treated with salt and used for the gene expression analysis [28]. qPCR analysis showed that *PvDOF* genes are expressed as a cultivar- or tissue-specific manner in common bean (Figure 5). Gene expression levels of *PvDOF-17, -27, -28* and *-30* genes were found to be higher in both tissues of 'Zulbiye' cultivar under salt stress compared to untreated control. Only *PvDOF-35* was found to be down-regulated compared to the control. However, it did not reach to a statistical significance ($p>0.05$). *PvDOF-27*, *PvDOF-28* and *PvDOF-17* genes exhibited the highest expression levels by 4.91-, 3.47- and 3.36- fold changes in the root tissue compared to its control in cv. 'Zulbiye', respectively. On the other hand, all genes except for *PvDOF-35* were expressed at least 1.5 fold higher in the leaves of cv. 'Zulbiye' than those of cv. 'Yakutiye' under salt stress conditions compared to their own untreated controls (Figure 5).

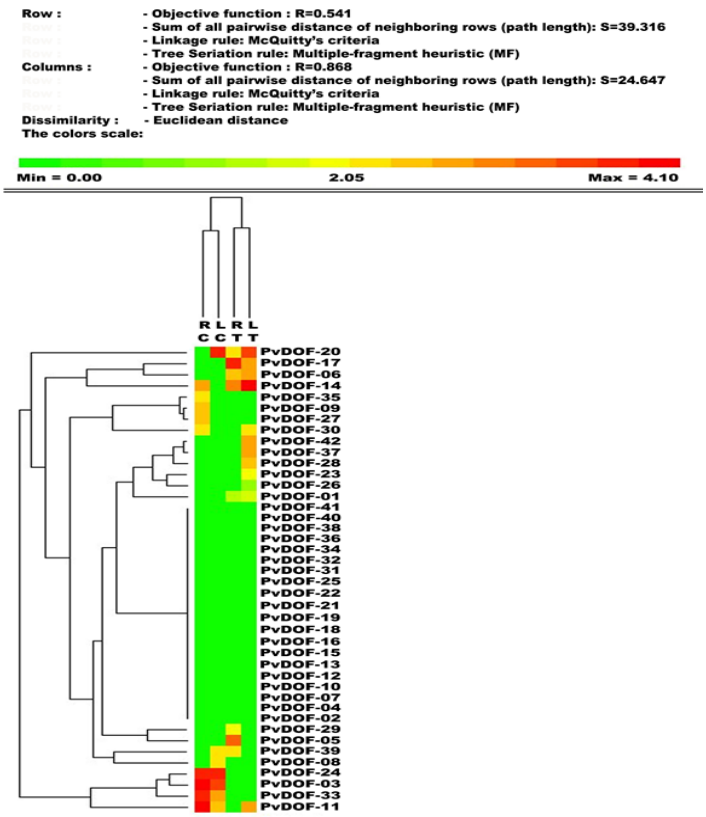

FIGURE 4. Heat map of differentially expressed *PvDOF* genes under normal and salt stress conditions. Figure was derived from Illumina RNA-seq data which was reported by Hiz et al. (2014) and downloaded from Sequence Read Archive (Accession numbers; SRR957667 (control leaf), SRR958472 (salt-treated root), SRR958469 (control root), and SRR957668 (salt-treated leaf) [26]. RT: Root treated, RC: Root control, LT: Leaf treated, LC: Leaf control.

PvDOF-17 gene was found to be down-regulated in leaf and root tissues of cv. 'Yakutiye', whereas it was up-regulated in both tissues of cv. 'Zulbiye' compared to the control under salt stress conditions. It might be concluded that the up-regulation of *PvDOF-17* gene in 'Zulbiye', which is known to be sensitive to osmotic stress compared to cv. 'Yakutiye', could be related with maximum effort of cv. 'Zulbiye' to deal with salt stress conditions. In addition, *PvDOF-17* was found to be targeted by miR854 according to miRNA analysis and miR854 was

also found to be responsive to osmotic stress in *Arabidopsis* and *Populus trichocarpa* Torr. & A.Gray ex Hook. previously [52].

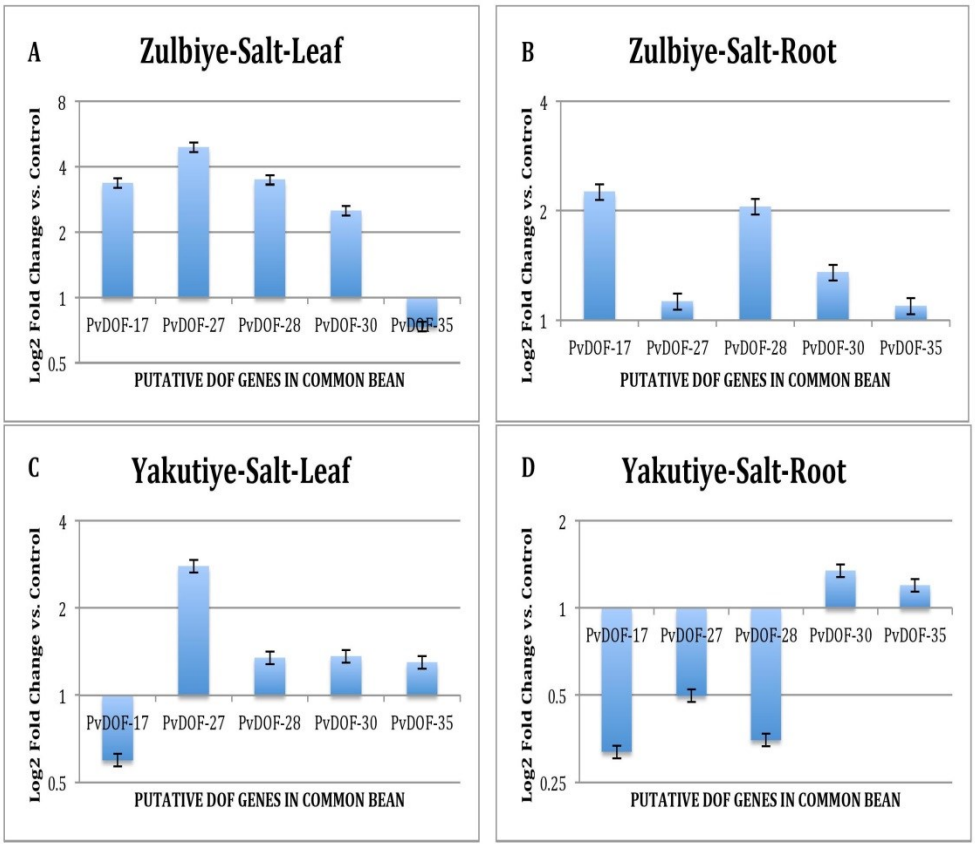

FIGURE 5. Expression patterns of *PvDOF* genes in leaves and roots of Yakutiye and Zulbiye bean cultivars under salt stress.

As a summary, in the current study we bioinformatically performed a genome-wide analysis of *PvDOF* transcription factor genes in the common bean genome. The identified 42 *PvDOF* genes were classified into eight subgroups and many properties of each PvDOF genes/proteins were characterized. The expressions of 9 out of 42 *PvDOF* genes were changed upon salt stress treatment according to the digital gene expression analysis. qPCR analysis of selected 5 *PvDOF* genes based on their RPKM values supported the results of the digital gene expression analysis.

4. Conclusions

We identified 42 putative PvDOF proteins, which were classified into four groups (A to D) in common bean genome and those DOF members were further characterized using bioinformatic analyses. The expression levels of putative PvDOF genes were also analyzed using publicly sequence read archive (SRA) and the expression levels of selected five PvDOF genes were confirmed through qPCR in two different common bean cultivars; cv. 'Yakutiye' and cv. 'Zulbiye' under salt stress conditions. The PvDOF35 gene was found to be down- and up-regulated in the leaf and root tissues of salt sensitive cv. 'Zulbiye', respectively. An opposite relationship was observed between sensitive cv. 'Zulbiye' and resistance cv. 'Yakutiye' under salt stress conditions with respect to gene expression levels of PvDOF17. Additionally, the most targeted genes were PvDOF-8 and -17, which were targeted by different plant miRNAs. In conclusion, this is the first study, which has a great value for providing gene expression data of PvDOFs against salt stress conditions. Therefore, this study might help the other researchers, who work in this field to understand the role of PvDOF in salt stress defense in common bean and would be used for future breeding programs in agriculture.

Acknowledgements This work was supported by Ankara University Scientific Research Coordination Unit. Project Number: 16H0430010. Additionally, some important parts of this study were used for oral presentation at '1st International Black Sea Congress on Environmental Sciences (1st IBCESS)' in 2016.

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