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THE NIN-LIKE PROTEIN (NLP) FAMILY IN COMMON BEAN: GENOME-WIDE IDENTIFICATION, EVOLUTION AND EXPRESSION ANALYSIS

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ABSTRACT. One of the plant-specific transcription factor families that play an important role in responses to nitrogen deficiency is NODULE INCEPTION-like (NIN-like) proteins (NLPs). However, the properties and evolutionary relationships of *NIN* genes in *P. vulgaris*, which enable nodule formation naturally, have not been studied yet. 12 *Pvul-NIN* genes have been identified in this study and the approximate positions of these genes have been determined. At the same time, several biochemical and physicochemical properties of NIN-like proteins have been elucidated in common bean. ORFs, which are the main coding regions of the *Pvul-NIN* genes, have been identified and were found to vary in length between 645-2976 bp. NIN proteins in the genome of *P. vulgaris* are 214-991 amino acids long and have a molecular weight of between 10.15-90.82 kDa. Comparisons between both monocot and dicot, but also nodule binding and non-nodule binding species were considered when investigating the evolutionary relationships of *NIN* genes. 16 duplication events (14 segmental and 2 tandem) have been shown to play a role in the expansion of the *NIN* gene family in *P. vulgaris*. In addition, comparative expression analysis of *NIN* genes was performed by processing publicly available RNAseq data and different levels of *Pvul-NIN* gene expression under both salt and drought stress were detected, suggesting the possible roles of *Pvul-NIN* genes for abiotic stress response. Expression levels of *Pvul-NIN* genes have also been investigated in different plant tissues and they have been shown to be intensely expressed in nodules and root tissues. This is the first study on the in-silico detection and characterization of *Pvul-NIN* genes to examine gene expression levels in common bean. The results could therefore provide the basis for future studies of functional characterization of *Pvul-NIN* genes.

Keyword and phrases. NIN-like proteins (NLPs), in-silico analysis, bioinformatics, bean, RNAseq

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1. Introduction

The basic component of proteins, chlorophylls, nucleotides and hormones is nitrogen (N), which has a significant influence on plant growth and productivity [1-4]. The growing population around the world is the need for food requirements and large quantities of N fertilizer are therefore being added to the soil to increase crop yield [5, 6]. The synthetic fertilizers used raise costs and are consumed in compliance with soil conditions and the form of plant [6-8]. Different adverse effects may be caused by this circumstance, such as an increase in the amount of nitrate in the water, soil surface degradation, contamination of water with chemical fertilizers, an increase in the amount of phosphate in rivers and the accumulation of harmful substances in plants due to excessive chemical fertilization. At the same time, greenhouse gases (such as N_2O) that contribute to climate change are produced by excess N input [9-11].

Therefore, it has become urgent that high crop yields should be matched with lower N fertilizer inputs and that the N efficiency of use (NUE) should be increased in order to preserve the soil, an indispensable habitat for all biological entities [10, 12-14]. Nitrate is one of the most common inorganic N types in aerobic soils, but it is the most readily permeable form of N because of its chemical structure [15]. While transgenic approaches are considered to be the most promising way to meet the current demand for high NUE crops, a well understanding of all N uptake and assimilation processes are needed [5]. Interaction of plants with microorganisms like rhizobia enhances the nutrient/fertilizer use efficiency. Newly shaped nitrogen fixing root nodules (NFN) use nitrogen from the atmosphere by symbiotic nitrogen fixation when legumes interact with rhizobia [16]. Symbiotic nitrogen fixation mechanisms which can directly fix nitrogen for crop improvement have been investigated thanks to the emerging mechanisms of genetic engineering [17].

Studies have shown that nitrate can act as a nutrient in plants as well as a signal molecule [18-20]. In line with the studies, components of the nitrate signaling pathway have been identified in recent years [19, 21]. RWP-RKs containing a preserved DNA binding pattern are a class of transcription factors (TFs) that control the efficiency of N uptake and N use by the detection of nitrate signals [22]. TFs are protein groups that play a role in the determination and functioning of genetic codes in DNA and in the regulation of gene expressions, and can also activate the transcription of the RNA polymerase gene and also affect the interruption of its transcription [23, 24]. This plant-specific TF family is divided into two classes:

nodule inception (NIN)-like proteins (NLPs) and RWP-RK-domain proteins (RKDs) [25]. When NLP and RKD were compared, it turned out that NLPs contain an additional domain known as PB1 (Phox and Bem 1) that allows interactions with additional proteins at their C-terminals [22]. When two studies on non-nodulating *Arabidopsis thaliana* are investigated, different effects of (NIN)-like proteins and RKDs on plants are detected; while RKDs are highly expressed in reproductive organs and play a regulatory role in female gametophyte development, (NIN)-like proteins play a central role in nitrate signaling by binding to nitrate-sensitive cis elements in target genes [26, 27].

(NIN)-like proteins also play important roles in the cross-interaction of the nitrate signaling pathway and the symbiotic signaling pathway as well as in the nodulation process. The *NIN* gene, which is functionally required for the formation of nodules, was first identified in *Lotus japonicus* [28]. The main factor in the formation of nodules is infection by microorganisms which varies depending on the plant's perception of N levels [29, 30].

Cultivated beans (*P. vulgaris* L.) belonging to the legume family, which are the main source of vegetable protein in nutrition, are of great importance to the world. Bacteria of *Rhizobium phaseoli*, which have a symbiotic relationship with the *P. vulgaris* roots, bind nitrogen that is free in the air but can not be used by living organisms directly [31]. Nitrogen, which is taken from the atmosphere in the root of the bean plant, accumulates in the nodules formed by *R. phaseoli*, is broken down by microorganisms after the bean is harvested and becomes an element [32].

Although the amount of nitrogen legumes bind to soil varies depending on the variety and environmental conditions, it is generally 5-19 kg/da per year. When the nitrogen that legumes bind to the soil is accepted as 10 kg/da per year, it corresponds to 50 kg of 20 per cent ammonium sulphate fertilizer. This means: nitrogen bonded to the soil by *Rhizobium* bacteria; lack of risk for washing, water pollution caused by excessive use of nitrogen fertilizers, low quality resulting from the use of artificial fertilizers and economic importance [33-37]. If, for the first time, a legume plant is to be planted in the soil in order to increase the chance of nodule formation in a young plant, the surface of the seed should be contaminated with a sufficient number of nodule bacteria unique to that plant before planting. This process is known as bacterial inoculation [38]. Inoculation of nitrogen-fixing bacteria, such as *Rhizobium leguminosarum*, *Rhizobium phaseali* and *Rhizobium japonica*, is a common cultural procedure [39, 40]. These bacteria enter the young roots only from

the tip of the root hairs of the legume plant in which they cohabit. When encountering a tetraploid cell in the edge tissue of the stem, these cells and the adjacent diploid

cells are stimulated to divide. During this period, the grafting pipes branch out and distribute to the tetraploid cells. Root tissue stimulated by bacteria and growth hormones develops abnormally and forms nodules. Cytokinins, one of the growth hormones of plants, provide the formation of nodules by activating the nodule genes and promoting cell division [41-43]. By rotting their roots, legumes release nitrogen into the soil. Organic substances with a high nitrogen content in the soil are known to decompose in a shorter time. Although the degradation period of the legume roots with a C/N ratio of 13:1 is 1-2 weeks under suitable conditions; it has been observed that this period was 4-8 weeks in the cereal roots with a C/N ratio of 80:1. Because the roots of edible legumes are protein-rich, there is adequate nitrogen in the soil, so the activity of microorganisms in the soil is faster. With all these characteristics, the legumes prepare suitable soil conditions for the plant to be planted in the soil after it has been planted [44, 45].

In this study, the functional roles and genome-wide analysis of NLPs in common bean were investigated using bioinformatics tools to deeply characterize *NIN* genes in a naturally nodulating plant. The function of *Pvul-NIN* genes was determined in the drought and salt response via RNAseq data. These results will provide insight into new studies on *P. vulgaris* or different plant species with *NIN* genes, improve current demand for NUE in crops, and guide the development of genetic engineering studies to provide a comprehensive understanding of all processes related to N uptake and assimilation and ensure that environmental damage caused by the use of chemical fertilizers is taken into account.

2. Materials and Methods

2.1. Identification of NIN-like proteins in *Phaseolus vulgaris* genome

P. vulgaris NIN family sequences were obtained from Phytozome v12.1 (http:// www.phytozome.net) and Pfam databases [46]. Putative *P. vulgaris* NIN-like proteins were used for query in blastp (NCBI) for characterization of hypothetical proteins. The physicochemical properties of NIN proteins were calculated using ProtParam Tool (http:web.expasy.org/protparam) and HMMER (http:www.ebi.ac.uk).

2.2. Structure and physical locations of *Pvul-NIN* genes and conserved motifs

Exon – intron structure of *Pvul-NIN* genes was represented using 'Gene Structure Display Server v2.0' (GSDS, http:// gsds.cbi.pku.edu.cn/) [47]. The *Pvul-NIN* genes have been mapped with MapChart on *P. vulgaris* chromosomes [48] Multiple EM for Motif Elicition method was used (MEME 4.11.1; http:/meme-suite.org/) to classify additional conserved motifs for Pvul-NIN proteins [49].

2.3. Phylogenetic analysis and sequence alignment

The ClustalW has been used to perform the multiple sequence alignment of Pvul-NIN proteins [50]. The Neighboring approach (NJ) was used for the construction of phylogenetic trees with a bootstrap value of 1000 replicates (MEGA7) and the tree was drawn using an Interactive Life Tree (iTOL; http://itol.embl.de/index.shtml) [51].

2.4. Promoter analysis of *Pvul-NIN* genes

Applying Phytozome database v11, the 5′ upstream regions (2 kb of DNA sequence from each *Pvul–NIN* gene) were analyzed with the CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/ html/) for a cis element scan.

2.5. In silico prediction of miRNA targets in *Pvul-NIN* genes

All known sequences of miRNA plants have been downloaded from miRBase v21.0. (http://www.mirbase.org). psRNA Target Server) was used accordingly with default miRNA prediction parameters (http://plantgrn.noble.org/psRNATarget) [52]. In-silico predicted miRNA targets were searched by BLASTX with $\leq 1e^{-10}$ against typical bean Expressed Sequenced Tags (ESTs) in the NCBI database.

2.6. Detection of gene duplication events and prediction of synonymous and non synonymous substitution rates

Segmental duplicate gene pairs were analyzed on the Plant Genome Duplication Database server (http://chibba.agtec.uga.edu/duplication/index/locus) with a display range of 100 kb. CLUSTALW software was used to predict amino acid sequences

of segmentally duplicated *Pvul-NIN* genes. The PAML (PAL2NAL) CODEML software (http://www.bork.embl.de/pal2nal) was used to estimate synonymous (Ks) and non-synonymous (Ka) substitution rates [53]. Duplication period (Million Years

ago, Mya) and divergence of each *Pvul-NIN* gene was calculated using the following formula: $T = Ks/2\lambda$ ($\lambda=6.56E^{-9}$) [54].

2.7. In-silico mRNA levels of *Pvul-NIN* genes in different tissues

Expression levels of *Pvul-NIN* genes in special tissue libraries of plants at different stages of development, including roots, nodules, young buds, stems, green mature buds, leaves, young triloliates, flower buds and flowers, were obtained from Phytozome Database v12.1. FPKM (expected number of fragments per kilobase of transcript sequence per million base pairs sequenced). FPKM values have been transformed log2 and a heatmap has been developed with the CIMMiner algorithm (http://discover.nci.nih.gov/cimminer).

2.8. Identified expression level of *Pvul-NIN* genes through transcriptome data

Illumina RNA-seq data was collected from the Sequence Read Archive (SRA) to measure the *Pvul-NIN* gene expression levels. For this reason, the accession numbers SRR957667 (control leaf), SRR958472 (salt-treated root), SRR958469 (control root) and SRR957668 (salt-treated leaf) were used as defined by Buyuk et al. (2016) [55]. The heat map of hierarchical clustering were eventually built using the PermutMatrix.

2.9. Homology modeling of Pvul-NIN proteins

All Pvul-NIN proteins were searched against Protein Data Bank (PDB) by BLASTP (with default parameters) to classify the best template(s) with identical sequence and three-dimensional structure [56]. Data were fed in Phyre2 (Protein Homology/AnalogY Recognition Engine; (http://www.sbg.bio.ic.ac.uk/phyre2) to predict protein structure by homology modeling in 'intensive' mode [57].

3. Results and Discussion

3.1. Identification of *Pvul-NIN* genes in the *Phaseolus vulgaris* genome

The 12 *NIN* genes *(Pvul-NIN)* defined for the *P. vulgaris* plant were named from *Pvul-NIN-1* to *Pvul-NIN-12* according to their chromosome order. ORFs, which are the main coding regions of the *Pvul-NIN* genes, have been identified and were found to vary in length between 645-2976 bp. NIN proteins in the genome of *P. vulgaris* are 214-991 amino acids long and have a molecular weight of between 10.15-90.82 kDa. It was determined that only two of the 12 Pvul-NIN proteins were basic and that the remaining 10 Pvul-NIN proteins were acidic. In addition, it was understood

by the determination of the instability indexes that they are all above 40 and therefore unstable.

The aliphatic index is defined as the relative volume of the aliphatic side chains (alanine, valine, isoleucine and leucine) which are considered to be a positive factor in the thermostability of globular proteins [46]. The aliphatic index of Pvul-NIN proteins range from 63.36 to 84.83. With the GRAVY value determined, it was observed that all Pvul-NIN proteins had negative values (-0.334 to-0.921) and therefore, Pvul-NIN proteins were hydrophilic.

At the same time, the predicted locations of the *Pvul-NIN* genes were examined and it was determined that all genes were located in the nucleus, albeit in different proportions. In addition to nucleus, it has been observed that *Pvul-NIN* genes may also be present in chloroplast, cytoplasm, extracellular matrix, vacuole and cytoplasmic skeleton at different rates.

The chromosomal distribution of *Pvul-NIN* genes in the *P. vulgaris* plant was investigated and therefore, 12 *Pvul-NIN* genes were distributed one by one to chromosomes 2, 3, 4, 5, 7, 8 and 11, and the remaining 5 *Pvul-NIN* genes were found to be on chromosome 9. (Figure 1). In a study conducted on *Brassica napus* (Canola), Liu et al (2018) found that 31 *NIN* genes are distributed to 15 chromosomes at different rates and contain only one gene for each of their 6 chromosomes, similar to *Pvul-NIN* genes [58].

TABLE 1. Descriptive information of Pvul-NIN proteins

ID	*Pvul-NIN-1*	*Pvul-NIN-2*	*Pvul-NIN-3*	*Pvul-NIN-4*	*Pvul-NIN-5*
***P. vulgaris* Genomic Database Identifier**	Phvul.002G116000.1	Phvul.003G189900.1	Phvul.004G114100.1	Phvul.005G155100.1	Phvul.007G071900.1
Chr.	2	3	4	5	7
Start position (bp)	24,790,146	41,337,458	39,383,865	38,469,742	6,724,515
End Position (bp)	24,795,421	41,339,39	39,388,798	38,480,117	6,729,082
Open reading frame/bp	999	816	2943	2937	2976
Protein length (aa)	332	271	980	978	991
pI	6.43	5.44	5.80	6.12	5.57
Molecular weight (Da)	38.22	31.72	10.93	10.76	10.98
Instability index	50.12	65.88	52.83	49.99	60.75
Aliphatic index	79.52	70.55	75.21	78.14	75.85
GRAVY	-0.651	-0.724	-0.452	-0.346	-0.421
NCBI Accession No.	XP_007157999.1	XP_007155308.1	XP_007152252.1	XP_007150469.1	XP_007143431.1
Predicted Location	nucl: 5, chlo: 4, cyto: 3, extr: 1	nucl: 11, chlo: 1, cyto: 1	nucl: 13	nucl: 7, cyto: 5, vacu: 1	nucl: 12, cyto: 1

TABLE 1. Descriptive information of Pvul-NIN proteins (continued)

Pvul-NIN-12	***Pvul-NIN-11***	***Pvul-NIN-10***	***Pvul-NIN-9***	***Pvul-NIN-8***	***Pvul-NIN-7***	***Pvul-NIN-6***
Phvul.011G052100.1	Phvul.009G186900.1	Phvul.009G115800.1	Phvul.009G080800.1	Phvul.009G051600.1	Phvul.009G011200.1	Phvul.008G291800.1
11	9	9	9	9	9	8
4,592,471	28,299,342	17,846,862	13,372,806	10,005,449	1,926,427	62,905,298
4,598,269	28,300,379	17,850,344	13,374,986	10,007,054	1,930,945	62,908,086
2907	645	2469	1671	810	2745	2178
968	214	822	556	269	914	725
5.60	8.79	5.75	4.79	8.13	5.79	6.12
10.67	25.15	90.82	62.79	30.65	10.15	80.52
46.70	56.47	45.25	65.43	52.68	53.88	51.95
75.51	63.36	76.48	79.21	84.83	74.46	73.41
-0.426	-0.921	-0.343	-0.612	-0.488	-0.478	-0.334
XP_007131923.1	XP_007138178.1	XP_007137300.1	XP_007136870.1	XP_007136514.1	XP_007136019.1	XP_007142568.1
nucl: 10, cyto: 2, vacu: 1	nucl: 13	nucl: 14	nucl: 10, cyto: 2, chlo: 1	nucl: 8, cyto: 3, cysk: 2	nucl: 13	nucl: 13

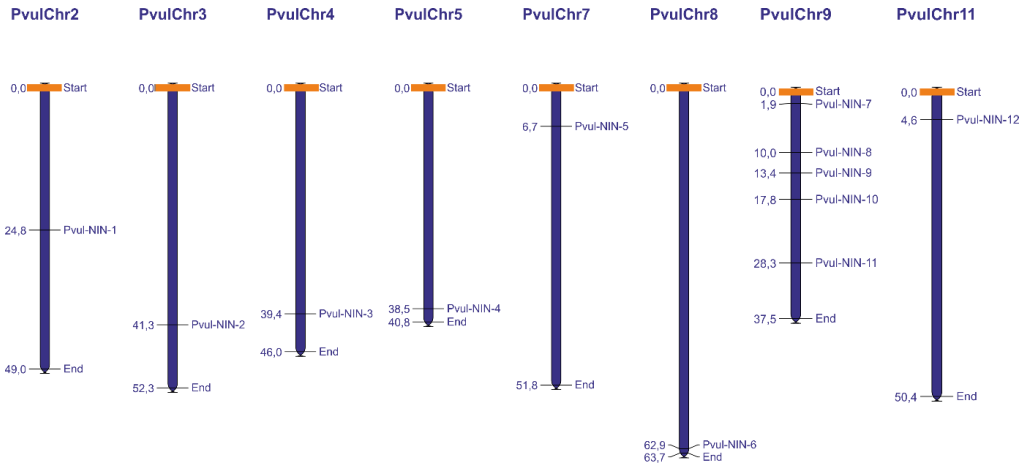

FIGURE 1. Distribution of *Pvul-NIN* genes on *P. vulgaris* chromosomes

Gene duplication generates a number of gene copies that can lead to the evolution of gene families [59]. Duplications may also contribute to the creation of new species by transcribing short chromosomal segments in the genome [60]. An analysis of the separation and duplication events of *Pvul-NIN* genes was conducted in this study. The ratios of homologous (Ks) and non-homologous (Ka) genes (Ka/Ks) were calculated for Darwin's Positive Selection Relationships and evaluated for *Pvul-NIN* genes [61]. A total of 14 pairs of segmental and 2 pairs of tandem duplicated genes have been identified among the *Pvul-NIN* genes (Table 2). On average, the Ka/Ks ratios are 0.06 for segmentally duplicated genes and 0.07 for tandemly duplicated genes. The estimated time of separation of the duplicated segmental *Pvul-NIN* genes varies between 13.21 and 455.24 million years (MYA), with an average of 205 MYA, while the average estimated time of separation of the tandemly duplicated *Pvul-NIN* genes is 247 MYA.

TABLE 2. Tandem - segmental duplication and Ka / Ks values seen in *Pvul-NIN* genes

Gene 1	Gene 2	Ks	Ka	Ka/Ks	MYA	Duplication Type
Pvul-NIN-2	*Pvul-NIN-11*	1,7177	0,3185	0,1854	13,21	Segmental
Pvul-NIN-3	*Pvul-NIN-4*	21,4659	0,7077	0,033	165,12	Segmental
Pvul-NIN-3	*Pvul-NIN-6*	7,6682	0,658	0,0858	58,98	Segmental
Pvul-NIN-3	*Pvul-NIN-7*	10,2981	0,6476	0,0629	79,21	Segmental
Pvul-NIN-3	*Pvul-NIN-12*	59,1812	0,7168	0,0121	455,24	Segmental
Pvul-NIN-4	*Pvul-NIN-5*	54,6548	0,6901	0,0126	420,42	Segmental
Pvul-NIN-4	*Pvul-NIN-7*	54,1101	0,6898	0,0127	416,23	Segmental
Pvul-NIN-4	*Pvul-NIN-10*	9,6954	0,7717	0,0796	74,58	Segmental
Pvul-NIN-5	*Pvul-NIN-6*	11,9819	0,6563	0,0548	92,16	Segmental
Pvul-NIN-5	*Pvul-NIN-7*	13,8683	0,6724	0,0485	106,67	Segmental
Pvul-NIN-5	*Pvul-NIN-12*	44,8914	0,6852	0,0153	345,31	Segmental
Pvul-NIN-6	*Pvul-NIN-7*	3,5771	0,5523	0,1544	27,51	Segmental
Pvul-NIN-7	*Pvul-NIN-10*	4,4037	0,5484	0,1245	33,8	Tandem
Pvul-NIN-7	*Pvul-NIN-11*	60,0494	1,1685	0,0195	461,91	Tandem
Pvul-NIN-7	*Pvul-NIN-12*	54,6615	0,6938	0,0127	420,47	Segmental
Pvul-NIN-11	*Pvul-NIN-12*	25,8255	0,9338	0,0362	198,65	Segmental

3.2. Structure of *Pvul-NIN* Genes, Phylogenetic Tree Analysis, Determination of Preserved Motifs, Homology Modeling and Promoter Analysis

At the same time, exon and intron structures of *Pvul-NIN* genes were determined

(Figure 2). The obtained exon-intron profiles contributed to the understanding of gene structure, and evaluation of motifs and phylogenetic relationships. Accordingly, the number of exons varies between 3 and 6, while the number of introns varies between 2 and 5. Similar to the *Pvul-NIN* genes, in a previous report by Cho (2017) on *Zea mays*, it was observed that the exon and intron structures of 9 *NIN* genes were found in similar numbers to the ones in this study, the number of exons varied between 4 and 5, while the number of introns varied between 3 and 4 [62].

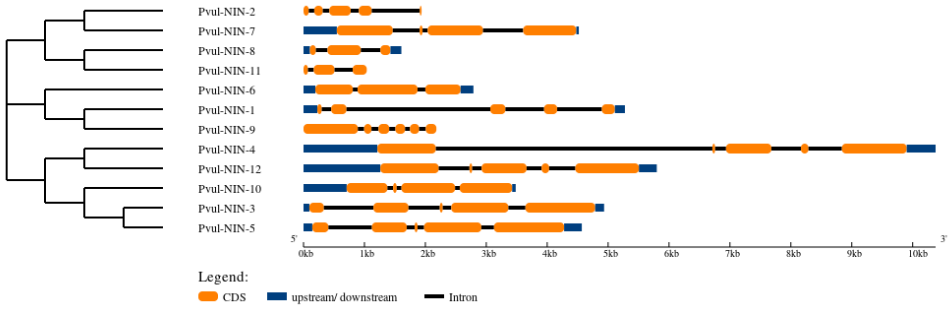

FIGURE 2. Exon intron structure of *Pvul-NIN* genes

Phylogenetic analysis was performed to understand and compare the evolutionary relationship between *Pvul-NIN* and *Arabidopsis* (non-nodulating) and *NIN* genes belonging to *G. max* (Nodulator). The phylogenetic tree formed with 54 NIN protein sequences was divided into three main groups (Figure 3). Followingly, Group A and B have 5 and 8 members respectively, while the C group with 41 members is the most crowded group. A phylogenetic tree analysis with 292 members including *P. vulgaris* as exogenous species and many different species was conducted in a study by Wu et al. (2020) on the effects of RWP-RK genes in *A. thaliana* on nitrate response [63]. The phylogenetic tree from that study reveals significant similarities with the tree drawn in the current study.

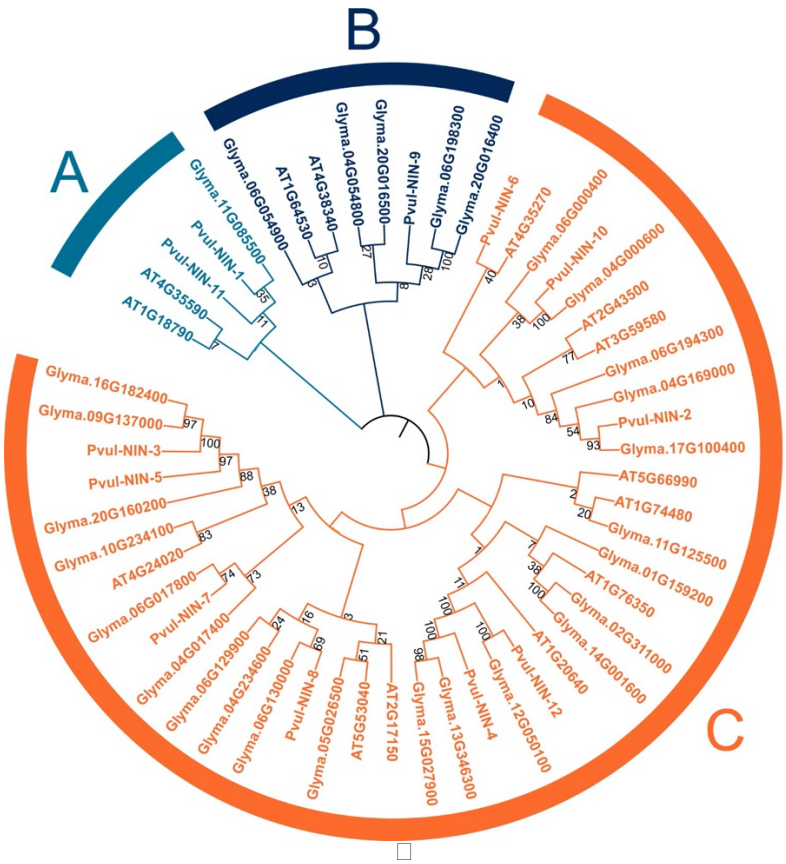

FIGURE 3. Phylogenetic classification of NIN-like proteins found in *Arabidopsis*, *G. max*, and *P. vulgaris.*

Short regions that are preserved in protein sequences and are closed to mutations are called as motifs [64]. The motif sequences of NIN-like proteins were found and the reliability of the phylogenetic analysis was checked and the profiles of the gene structure were examined (Figure 4). There is only one gene *(Pvul-NIN-9)* in the phylogenetic tree that belongs to *P. vulgaris* in group B. Only one preserved region of the *Pvul-NIN-9* gene was detected when the motif compositions were examined. This makes the gene of *Pvul-NIN-9* different from other genes.

There is an initiation transcription site within the genes. With reference to the transcription initiation site, the DNA region towards the 5' end of the coding strand is known as upstream DNA. A gene promoter is normally found upstream of the

DNA. The DNA region from the +1 point to the 3' end of the coding strand is known as the downstream DNA [65]. The upstream and downstream structures of *Pvul-NIN-2* and *Pvul-NIN-11* were not able to seen when the gene structure was examined. Another similarity in the same genes is that they have exactly the same motifs.

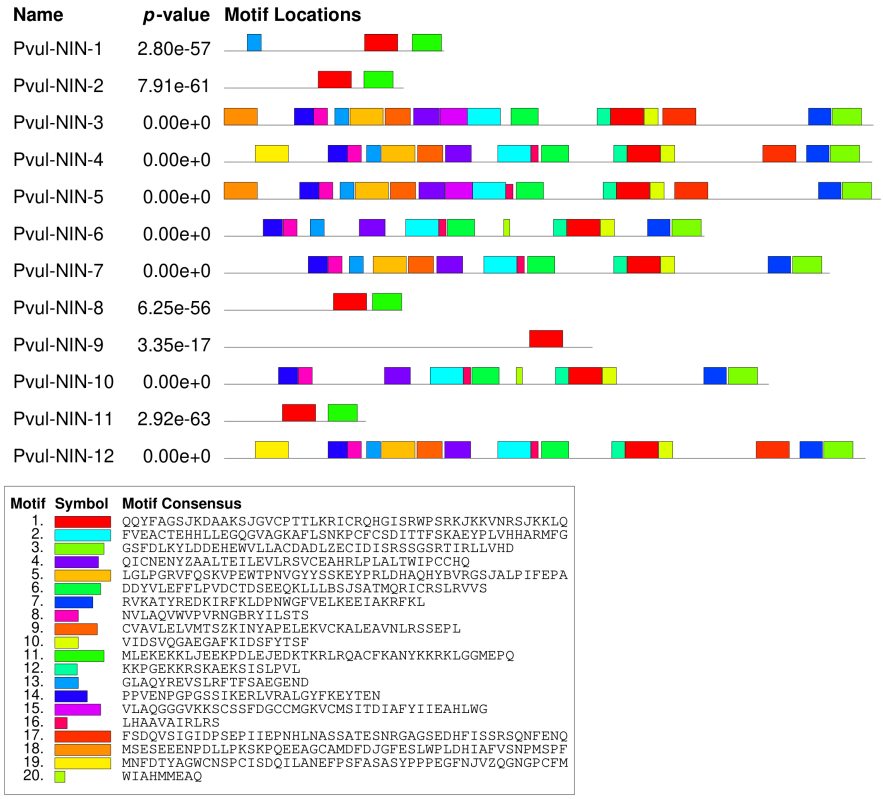

FIGURE 4. Motif contents of Pvul-NIN proteins

Homology modeling of NIN-like proteins of *P. vulgaris* was also performed with this study. Homology modeling is the process of analyzing the three-dimensional (3D) shape of a protein over one or more protein structures that have been structurally analyzed [66]. Models created by this method are related to the similarity of the sequences to each other [67]. 3D structure of Pvul-NIN-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12 proteins were extracted by homology modeling accordingly (Figure 5). The secondary protein structures express the local conformation of certain parts of the polypeptide. Stable and commonly found ones are α-helix and β-

conformations [68]. In Pvul-NIN proteins, α-helix forms a general structure. Alpha helix and β-layers may coexist as in Pvul-NIN-3,-4,-5,-6,-7,-8,-10,-12 proteins. This situation occurs with some important folding rules for the definition of simple motifs. The embedment of hydrophobic amino acid R groups to exclude water requires a secondary structure with at least two layers; the inner R groups form the Beta-alpha-beta ring (β- α- β Loop) and alpha-alpha (α- α corner) corner. These two structures are also observed in Pvul-NIN proteins.

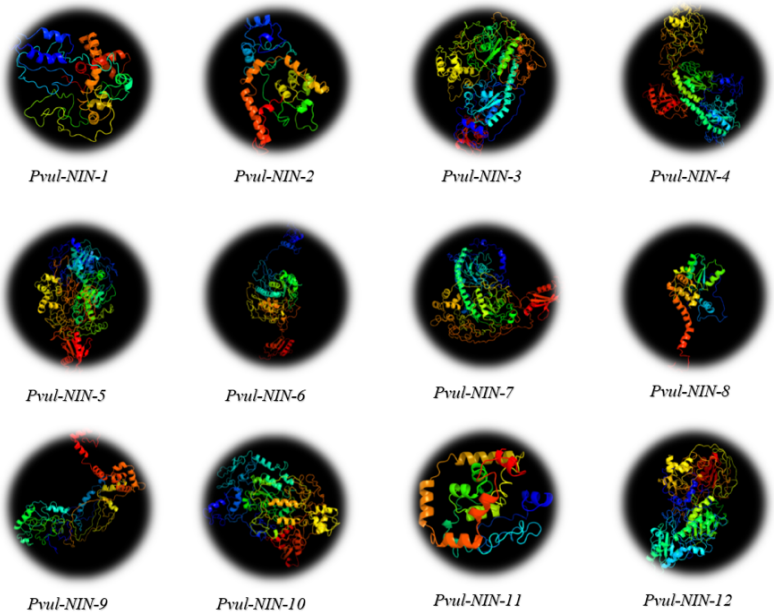

FIGURE 5. Predicted 3D structures of Pvul-NIN proteins

P. vulgaris NIN genes are primarily duplicated by segmental duplication rather than tandem duplication. It has been observed that there is a common duplication mechanism, although the duplication rate of the *Pvul-NIN* genes is lower than that of *Arabidopsis* and *G. max*. In order to examine orthological relationships in more detail, all *RWP-RK* genes have been examined, a synteny analysis including not only *NIN* genes but also RKD genes has been performed. Some orthological and chromosomal reconstructions have been observed compared to other species (Figure 6).

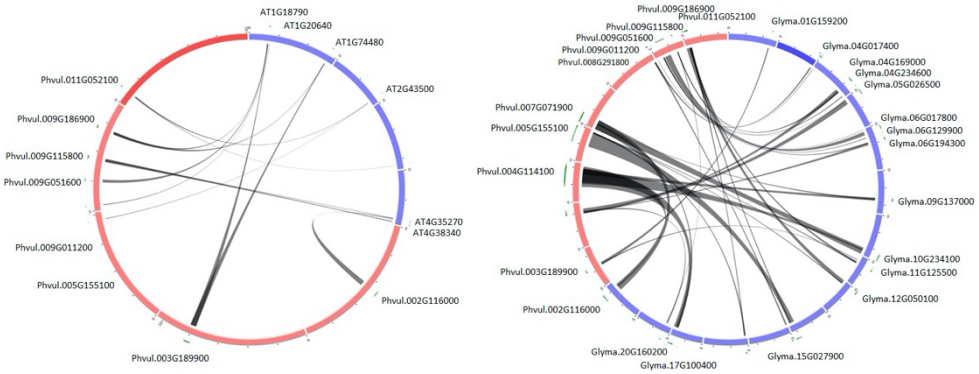

FIGURE 6. Orthologous relationship between *P. vulgaris-A. thaliana* and *P. vulgaris-G. max*

Cis-regulatory elements, vital components of genetic regulatory networks, are non-coding regions of DNA that regulate the transcription of neighboring genes [69]. In this study, in-silico promoter analysis was conducted to better understand the regulatory mechanisms of the *Pvul-NIN* genes. Identified cis-acting elements were grouped into 8 groups, including development, environmental stress, hormone, light, promoter, site binding, biotic stress and others (Supplementary Table 1).

CAAT-box and TATA-box, which are the main promoter elements, have been defined in all *Pvul-NIN* genes, as expected. Certain metabolites play an important role in the regulation of enzymes involved in nitrogen assimilation. In a previous report by Schiavon et al. (2008) on the use of inorganic N fertilizers in agriculture on *Zea mays*, the expression of enzymes involved in the tricarboxylic acid cycle (TCA) and nitrogen metabolism was investigated, and it was revealed that both were definitely related to plant productivity. In addition, as a result of their study, it has been shown that transcript accumulation is mainly root-induced. The presence of the TCA-element in the hormone category in all *Pvul-NIN* genes is remarkable given the nodule binding closely related to plant growth hormones [70].

Ethylene-Response Element (ERE), which is linked to the AGCCGCC core sequence (also known as the GCC box) to provide resistance to biotic and abiotic stress, is found in approximately 66% of *Pvul-NIN* genes [71]. In a study conducted

on MYB and MYC, which are present in approximately 83% of the *Pvul-NIN* genes, it has been shown that both cis-regulators on *Arabidopsis* have effects on drought

stress and act as transcriptional activators in ABA-inducible expression in the genes studied [72]. At the same time, the fact that Unnamed 4, the functions of which are not yet known, are included in all *Pvul-NIN* genes has revealed that it is a cis-regulatory that needs to be worked on.

3.3. Detection of miRNAs Targeting *Pvul-NIN* Genes

miRNAs complement the coding sequences (CDS) or non-translational regions (UTR) of the target mRNAs for post-transcriptional editing [73]. A total of 64 *Pvul-NIN* associated miRNAs were identified in this study as a result of miRNA analysis (Supplementary Table 2). Approximately 12% of the miRNA targeting the *Pvul-NIN* genes is miR172 which is known to be expressed during nodule formation in legumes, especially in root infestations of bacteria. In a previous study by Holt et al. (2015) on *Lotus japonicus*, it has been shown that miR172 plays an important role in the early stages of legume infection with rhizobial bacteria. MiR172 is strongly regulated at the early stages of symbiosis and expression precedes the progression of the infection. The study also showed that miR172 regulates the *NIN* genes in *Lotus japonicus* [74]. Approximately 20% of the miRNA targeting the *Pvul-NIN* genes is miR167. It is known that miR167 plays a central role in the maternal control of seed development. A study conducted on miR167, which is also closely related to auxin hormones, has shown that it is a dominant regulator in the reproduction of *A. thaliana* [75]. miR396 was also found to target *Pvul-NIN* genes and studies have shown that it is effective in the formation of adaptive responses such as growth of plants, hormonal signaling and leaf development under abiotic stress [76, 77]. miR902 has been shown to play a role in the regulation of plant endurance characteristics under drought stress [78]. miR185 changes the levels of expression in eukaryotic organisms under oxidative stress [79]. The gene most targeted by miRNAs with a rate of 22% is *Pvul-NIN-5* and it is followed by *Pvul-NIN-4* gene with 17%.

3.4. Tissue-specific mRNA levels of *Pvul-NIN* genes

Heat map revealed that *Pvul-NIN* genes give high levels of expression in different tissues such as flower buds, flowers, leaves, stem 10, young pods, stem 19, young trifoliates, root 10, root 19, green mature pods and nodules (Figure 7). Expression levels of all *Pvul-NIN* genes, but especially *Pvul-NIN-3,-4,-5,-6,-7,-10* and *-12* genes, have also been found to be high in nodules and root tissues.

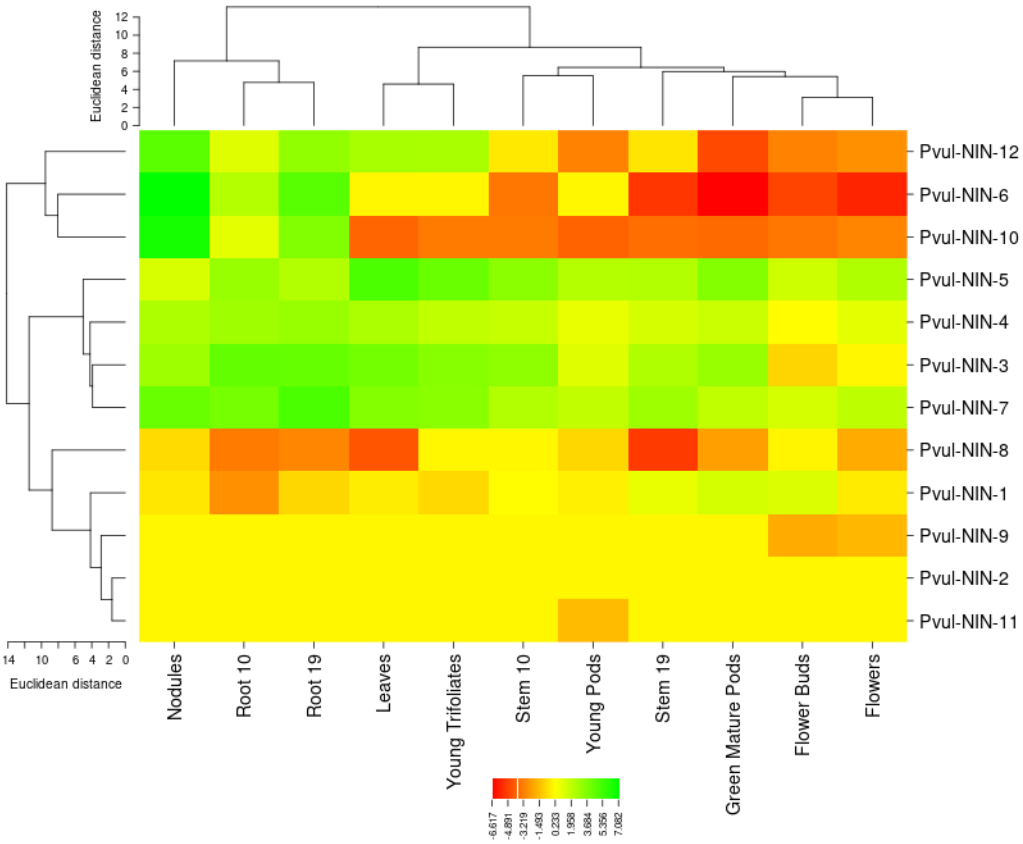

FIGURE 7. Heat map image for tissue specific mRNA levels of *Pvul-NIN* genes

3.5. Responses of *Pvul-NIN* Genes to Salt Stress and Drought Stress Through RNAseq Analysis

The RNAseq data generated by Hiz et al. (2014) and taken from GenBank (Hiz, Canher et al.) were analyzed to investigate the expression profiles of *Pvul-NIN* genes against abiotic stress such as drought and salt (Figure 8). As a result of these analyzes, changes in the expression of leaf tissue under salt and drought stress were identified. Expression levels of *Pvul-NIN-3,-4,-5* and *-7* genes were high in all

conditions but at varying degrees, and expression levels of *Pvul-NIN-8,-9* and *Pvul-NIN-11* decreased at different rates under drought stress. An increase in the

expression level of the *Pvul-NIN-12* gene under salt and drought stress was observed, while the expression levels in the *Pvul-NIN-6* and-*10* genes were decreased under both stresses. The changes in expression of some *Pvul-NIN* genes in response to salt and drought stresses may explain their involvement in stress signaling mechanisms in common bean and these findings should be verified with additional gene expression analyses in the future.

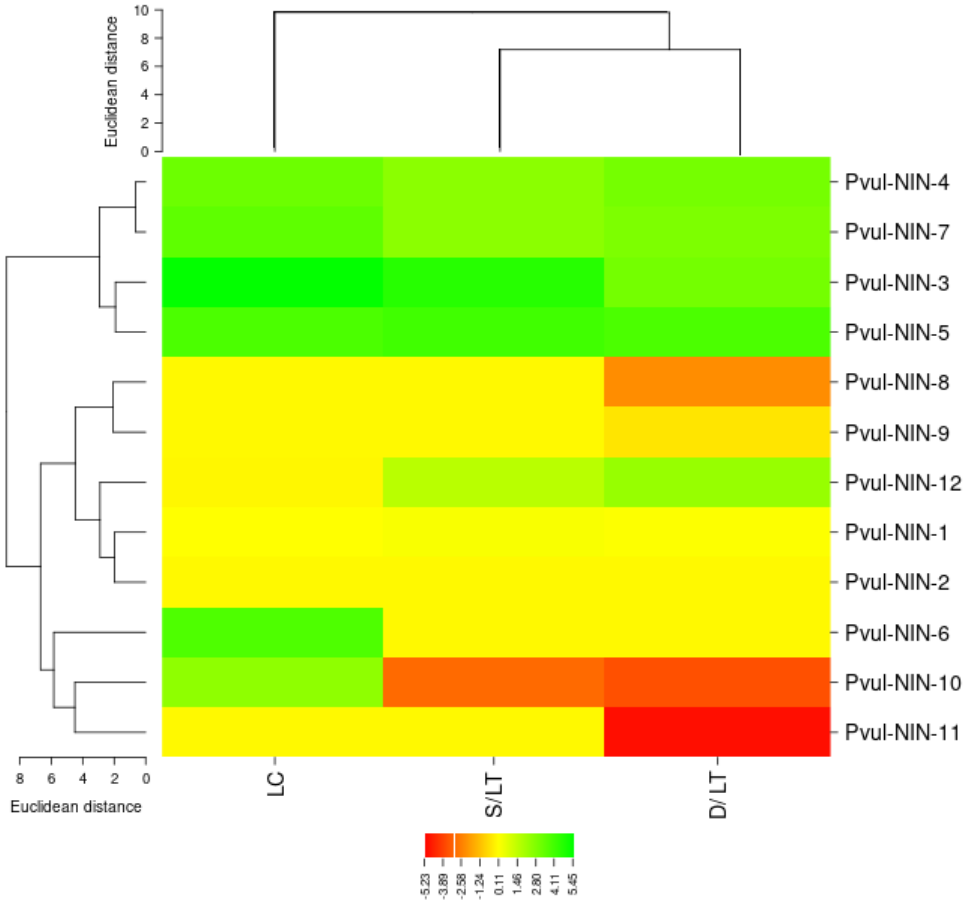

FIGURE 8. Heatmap of *Pvul-NIN* genes differentially expressed under normal, salt and drought stress conditions derived from RNAseq analysis

4. Conclusion

In addition to the negative effects of global warming, which is threatening the world as a whole, agricultural products are being damaged by biotic stress and abiotic stress factors such as drought and salinity, where agricultural areas are most damaged today, lowering quality and productivity, leading to product losses in advanced stages. In addition to all of these, excessive and incorrect fertilizer applications negatively affect the environment and cause permanent damage to soil and water. For these reasons, ensuring food and environmental safety, improving the use of N use efficiency (NUE) requires plants to increase resistance to stress factors through molecular studies. Legumes have *NIN* genes with functional responsibility for symbiotic nitrogen fixation. In this context, our study includes a genome-wide analysis of 12 NIN-like *P. vulgaris* proteins, which naturally allow the formation of nodules. The identified 12 *Pvul-NIN* genes are listed from the 2nd chromosome to the 11th chromosome. In order to understand the biological functions of the *NIN* genes in the *P. vulgaris* genome, a number of bioinformatics tools and databases have been used for various analyzes. Identifying NIN-like proteins (NLPs) for the first time in *P. vulgaris* and examining their reactions to various abiotic stress conditions, an analysis of miRNAs targeting hormones and *Pvul-NIN* genes that play an active role in the formation of nodules will shed light on a number of molecular studies to be conducted on fertilizer signaling pathways.

Authors Contribution Statement Experiment design: I.B., S.A., T.A., Experiment performance and data analyses: I.B., A.O., T.A., Data interpretation: I.B., A.O., T.A., S.A. and Manuscript drafting: I.B., A.O., T.A., S.A.

Declaration of Competing Interests The authors declare that they have no conflict of interest.

Supplementary Files:

Table1. https://dergipark.org.tr/en/download/article-file/1538060

Table2. https://dergipark.org.tr/en/download/article-file/1538057

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