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EIN3/EIL (Ethylene Insensitive3 / Ethylene Insensitive3 Like) Protein Family in *Phaseolus vulgaris*: Identification, Evolution and Expression Analysis within the Genome

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ABSTRACT

Ethylene insensitive-3 (EIN3) / Ethylene insensitive-3-like (EIL) protein family is a small family of transcription factors specific to plants that play role in plant growth and development under various environmental conditions. In this study, various bioinformatics approaches were used to make an in-depth identification of the EIN3/EIL family at both the gene and protein levels. So, 11 *Pvul-EIL* genes were identified and their approximate locations were determined. Various biochemical and physicochemical properties of EIL proteins in *Phaseolus vulgaris* have been described. It was determined that Pvul-EIL proteins had a length of 447-651 amino acids and a molecular weight of 51.08-70.68 kDa. All duplications occurring in the *Pvul-EIL* genome were segmental type. It was observed that conserved motif, gene structure and phylogeny analyses all yielded similar results. For instance, it has been understood that genes with same motif type and number have similar gene structures and were located under the same branch in the phylogenetic tree. Pvul-EIL protein homology modeling showed that DNA binding properties and protein structure were similar to Arabidopsis EIN3. According to cis-element analysis, *Pvul-EIL* genes are engaged in a wide range of functions, including tissue-specific, stress, and hormone-sensitive expression. Additionally, RNAseq data was used to perform a comparative expression analysis of *EIL* genes. Various *Pvul-EIL* gene expression levels were detected under salt and drought stress. This is the first study to check the gene expression levels in *P. vulgaris* using *in-silico* detection and characterization of *EIL* genes. Therefore, obtained results can form the basis for future studies.

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Introduction

Hormones (phytohormone-plant growth regulators) are spontaneously formed organic molecules. These organic molecules are transferred from their source to other parts of the plants in which they show their effects and can show their impact even at very low concentrations (Öktüren and Sönmez 2005). Ethylene is the smallest gaseous phytohormone with a simple chemical structure (C_2H_4) (Chao et al., 1997). It is involved in vital functions of the plant such as cell expansion and division, leaf growth, flower development, seed germination, organ senescence, root formation, sex determination and fruit ripening (Abeles et al., 2012). Ethylene also has a role in determining cell status in the root epidermis, signaling pathogen-associated systemic activation of defense genes and the wound response and formation of nitrogen-fixing nodules (Chao et al., 1997). As a result, the plant plays an important function in both the control of ethylene production and the capacity of cells to detect the hormone and react correctly to external stimuli (Chao et al., 1997). Since ethylene is generated in response to biotic and abiotic stressors such as flood, injury, heat, cold, starvation, salt stress, and pathogen infection, it serves a variety of functions in stress defense (Ju and Chang 2015; Li et al., 2019). Furthermore, ethylene can function as a signaling molecule and alter the mRNA levels of some genes (Lee and Kim 2003). Etiolyzed seedlings show a triple response to ethylene. This triple response includes three different morphological changes: hypocotyl elongation, horizontal stem growth, increased radial expansion (Guzman and Ecker 1990).

The ethylene insensitive-3 (*EIN3*) / Ethylene insensitive-3-like (*EIL*) family is a gene family specific to

advanced plants which includes well conserved amino acid sequences at N-terminal and proline-rich domains (Wawrzyńska and Sirko 2014). When the amino acid sequences at the C-terminus are compared to those at the N-terminus, it's observed that the C-terminus is less conserved. This may indicate that functional differences in the EIL family are determined by the aminoacid sequences at C-terminal (Rieu et al., 2003). EIN3/EIL proteins are key regulators that bind to primary ethylene response elements (PERE) and EIL conserved binding sequences (ECBS) involved in the ethylene reaction to initiate ethylene-mediated downstream transcriptional cascades (Liu et al., 2019). EIN3 / EIL proteins can induce the biosynthesis of some hormones by interacting with various plant growth regulators such as ethylene, gibberellin, jasmonic acid and salicylic acid (Chang 2016; Song et al., 2018; Zhu et al., 2011). An example of the involvement of EIL proteins is cross-talk in deepwater rice, where ethylene signaling increase gibberellins that signal internode elongation and then allow rice plants to escape complete submersion. (Bailey-Serres et al., 2010).

Although EIN3 and EIL1 control the ethylene response through distinct routes, they act together. While EIL1 limits leaf and stem development in adult plants, it has been determined that EIN3 enhances ethylene responses in Arabidopsis (An et al., 2010). Additionally, there have been studies on rice, tobacco, tomato, cucumber, and banana about EILs (Bie et al., 2013; Chao et al., 1997; Chen et al., 2004; Hiraga et al., 2009; Jourda et al., 2014; Li et al., 2012; Mao et al., 2006; Maruyama-Nakashita et al., 2006; Rieu et al., 2003; Wawrzyńska et al., 2010; Yang et al., 2015). According to studies on Arabidopsis, cloves, and mung beans, regions enriched with asparagine or glutamine are broadly dispersed across the C-terminal sequences of EIN3 / EIL members (Lee and Kim 2003; Waki et al., 2001). In contrast to previous studies, it was discovered that asparagine or glutamine-rich areas are rare in *NtEIL* members C terminal sequences (Rieu et al., 2003). In a study on kiwi, *EIL* genes were upregulated in low temperatures and in this way reacted to the stress response (Yin et al., 2009). It was thought that in case of high expression of *LeEIL* genes in tomato, they could positively regulate the ethylene response and fruit development (Tieman et al., 2001). Plants treated with exogenous ethylene, such as Arabidopsis, tobacco, mung bean, and tomato, were shown to have no effect on *EIL* gene expression (Lee and Kim 2003; Rieu et al., 2003; Tieman et al., 2001). Contrary to these studies, the expression of *EIL* genes was induced by exogenous ethylene application in plants such as rice, banana, clove and petunia (Iordachescu and Verlinden 2005; Shibuya et al., 2004). Yamasaki et al., (2005) identified DNA-binding domains (DBD), which is a V-shaped slit formed by five α-helices, in a study on EIL3 in *Arabidopsis thaliana*. It has been found that this conserved DBD is also found in all EIN3 homologs of other studied plant species and that EIN3/EIL DBD specifically binds to the EIN3 binding site (Yamasaki et al., 2005). According to the studies of Hiraga et al., (2009) and Yang et al. (2015) on wound signal and salt tolerance in rice, EIL family is found to be involved in the regulation of these two factors (Hiraga et al., 2009; Yang et al., 2015).

Phaseolus vulgaris, with a diploid chromosome number of 2n=22, is a species originated in Central America, which was cultivated in Peru around 5000 BC and in Southern Mexico around 6000 BC (Bitocchi et al., 2012; Campeanu et al., 2005; Zewdie and Hassen 2021). It has great importance in human nutrition with the proteins, vitamins and minerals it contains (Büyük et al., 2019). In addition to these features, its cheapness and easy preservation have made the bean a food with high consumption worldwide (Büyük et al., 2021b). Abiotic stress factors (salinity, drought, and low temperature) are said to impair agricultural yield and production by 15% globally, whereas biotic stress factors (bacterial, fungal, and viral threats) are said to damage crop yield and production by 82 % globally (Chaerle et al., 2007). These stress factors seriously damage the crop and production yield of beans. Salt stress, which is considered as one of the abiotic stress factors, has been identified as one of the most critical limiting factors in bean development (Ashraf 1999). Increasing the yield depends on determining and cultivating varieties suitable for ecological conditions and making the varieties resistant to these conditions in biotechnological applications. For this reason, it is important for plant biotechnology studies to elucidate the defense mechanisms of plants against environmental stress factors and to identify stress-related genes.

Materials and Methods

EILs in Phaseolus vulgaris

P. vulgaris EILs sequences were acquired from Phytozome v13 (https://phytozome-next.jgi.doe.gov/) using Pfam ID (PF04873) (Goodstein et al., 2012). For the characterization of hypothetical proteins, putative *P. vulgaris* EILs was queried in blastp (NCBI). The ProtParam tool was used to calculate the physicochemical properties of EILs (https://web.expasy.org/protparam/) and HMMER (https://www.ebi.ac.uk/).

EIL Gene Structure and Conserved Motifs

The *Pvul-EIL* genes' structure was represented using the 'Gene Structure Display Server v2.0' (GSDS, http://gsds.cbi.pku.edu.cn/) (Guo et al., 2007). MapChart was used to map the *Pvul-EIL* genes on *P. vulgaris* chromosomes (Voorrips 2002). To classify conserved motifs for Pvul-EILs, the Multiple EM for Motif Elicitation method (MEME 4.11.1; https://meme-suite.org/) was used (Bailey et al., 2006).

Phylogenetic Analysis and Sequence Alignment

ClustalW was used to perform sequence alignment of Pvul-EILs (Tamura et al., 2011). The Neighboring approach (NJ) was used to build phylogenetic trees with a bootstrap value of 1000 replicates (MEGA V11), and the tree was drawn using an Interactive Life Tree (iTOL; http://itol.embl.de/index.shtml) (Letunic and Bork 2011).

Promoter Analysis of Pvul-EIL Genes

The Phytozome database v13 was used to extract 2 kb upstream sequence of the *Pvul-EIL* genes. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to screen the cis-elements of the promoter regions.

In Silico Analysis of miRNAs Targeting Pvul-EIL Genes

miRBase v21.0 was used to obtain all known miRNA plant sequences. (http://www.mirbase.org). Moreover, psRNA Target Server (http://plantgrn.noble.org/psRNATarget) was used in its default form (Zhang 2005). BLASTX with $\leq 1e^{-10}$ versus common bean Expressed Sequenced Tags (ESTs) in the NCBI database was used to identify in silico suggested miRNA targets.

Estimation of Synonymous and Nonsynonymous Substitution Rates and Identification of Gene Duplication Occurrences

The Plant Genome Duplication Database site (http://chibba.agtec.uga.edu/duplication/index/locus) found segmentally duplicated gene pairs with a display range of 100 kb. ClustalW software was used to identify the amino acid sequences of segmentally duplicated *Pvul-EIL* genes. The synonymous (Ks) and non-synonymous (Ka) substitution rates were estimated using PAML (PAL2NAL) CODEML tool (http://www.bork.embl.de/pal2nal) (Suyama et al., 2006). T=Ks/2 (=6.56E9) was used to calculate the duplication period (Million Years Ago, MYA) and divergence of each *Pvul-EIL* gene (Yang and Nielsen 2000).

In Silico mRNA Concentration of Pvul-EIL Genes in Various Tissues

The Phytozome Database v13 was used to acquire expression levels of *Pvul-EIL* genes in particular tissue libraries of plants at various phases of development. In silico expression levels were calculated using FPKM (expected number of fragments per kilobase of transcript sequence per million base pairs sequenced), then FPKM data were log2 converted and a heatmap was created using the CIMMiner tool. (http://discover.nci.nih.gov/cimminer).

Determine Expression Level of Pvul-EIL Genes by the Help of Transcriptome Data Illumina

Pvul-EIL gene expression levels under salt and drought stresses were measured using RNAseq data from the Sequence Read Archive (SRA). So, the following accession numbers defined by Büyük et al., (2016) were used for salt and drought stresses: SRR957667 (control leaf for salt experiment), SRR957668 (salt-treated leaf), SRR8284481 (drought-treated leaf), and SRR8284480 (control leaf for drought experiment) (Büyük et al., 2016; Hiz et al., 2014; Jorge et al., 2020). The heatmaps of hierarchical clustering were achieved by the help of CIMMiner database (http://discover.nci.nih.gov/cimminer). Since the SRA data were collected from two separate experiments (salt and drought studies) by two different research groups, their control data were also diverse, resulting in two heatmaps.

Homology Modeling of EIL Proteins

All Pvul-EIL proteins were blasted against the Protein Data Bank (PDB) by BLASTP (with default parameters) to classify the best template(s) with identical sequence and 3D structure (Berman et al., 2000). The tertiary structure of EIL proteins in *P. vulgaris* was predicted by the help of homology modeling method (SWISS-MODEL, https://www.swissmodel.expasy.org) (Waterhouse et al., 2018).

Table 1. Detailed information on members of the *P. vulgaris* L. EIL family

ID	Genomic Database Identifier	Physical position on P. vulgaris genome			Protein length (aa)	pI
		Chr	Start Position (bp)	End Position (bp)		
Pvul-EIL-1	Phvul.001G051300	1	5,570,532	5,574,219	643	7.60
Pvul-EIL-2	Phvul.001G251100	1	50,162,187	50,163,531	447	4.98
Pvul-EIL-3	Phvul.002G253900	2	42,570,666	42,572,076	469	5.00
Pvul-EIL-4	Phvul.003G165500	3	38,522,178	38,525,896	651	6.34
Pvul-EIL-5	Phvul.005G012200	5	1,045,457	1,048,898	559	6.66
Pvul-EIL-6	Phvul.006G011200	6	5,048,237	5,051,367	619	5.49
Pvul-EIL-7	Phvul.006G011300	6	5,250,565	5,253,843	624	5.67
Pvul-EIL-8	Phvul.008G207000	8	55,496,792	55,500,388	611	5.50
Pvul-EIL-9	Phvul.009G080700	9	13,358,386	13,361,717	601	7.59
Pvul-EIL-10	Phvul.009G093200	9	14,831,097	14,834,639	643	8.06
Pvul-EIL-11	Phvul.L011743	scaffold	818,924	822,398	592	5.99

ID	Molecular weight (kDa)	Instability index	NCBI Acc. No	GRAVY	Aliphatic Index	Subcellular loc.
Pvul-EIL-1	68.22	38.52	XP_007161212.1	0.186	104.01	nucl: 10.5
Pvul-EIL-2	51.08	54.12	XP_007163637.1	-0.649	82.91	nucl:11
Pvul-EIL-3	54.09	57.51	XP_007159632.1	-0.799	74.46	nucl:11
Pvul-EIL-4	69.75	53.22	XP_007155011.1	0.233	109.92	nucl: 2
Pvul-EIL-5	63.13	45.01	XP_007148764.1	-0.650	65.38	nucl:12
Pvul-EIL-6	70.43	44.57	XP_007146083.1	-0.729	63.18	nucl:12
Pvul-EIL-7	70.68	42.56	XP_007146084.1	-0.689	64.50	nucl:14
Pvul-EIL-8	69.33	46.65	XP_007141570.1	-0.718	63.01	nucl:12
Pvul-EIL-9	64.85	43.48	XP_007136869.1	0.090	96.51	nucl: 9
Pvul-EIL-10	68.95	44.82	XP_007137020.1	0.115	99.30	nucl: 8.5
Pvul-EIL-11	66.53	57.88	XP_007150399.1	-0.845	69.09	nucl:12

Table 2. *Pvul-EIL* genes that are involved in segmental duplication events.

Gene-1	Gene-2	Ks	Ka	Ka/Ks	MYÖ	Duplication Type
Pvul-EIL-1	Pvul-EIL-10	0.84	0.21	0.25	6.46	Segmental
Pvul-EIL-2	Pvul-EIL-3	1.18	0.3	0.26	9.07	Segmental
Pvul-EIL-4	Pvul-EIL-10	25.15	0.34	0.13	193.46	Segmental
Pvul-EIL-5	Pvul-EIL-8	55.28	0.56	0.01	425.23	Segmental
Pvul-EIL-6	Pvul-EIL-8	1.3	0.17	0.13	10	Segmental

Table 3. Orthologous relationships identification of *EIL* genes between the *P. vulgaris* and *G. max* genomes

ID	Gene ID	Ks	Ka	Ka/Ks	MYA
Pvul-EIL-1	Glyma.06G068400	0.75	0.18	0.24	5.77
Pvul-EIL-1	Glyma.04G066900	0.72	0.17	0.24	5.60
Pvul-EIL-1	Glyma.14G116800	0.33	0.05	0.15	2.56
Pvul-EIL-1	Glyma.17G113900	1.81	0.31	0.17	13.98
Pvul-EIL-1	Glyma.17G211000	0.33	0.07	0.21	2.60
Pvul-EIL-2	Glyma.08G137800	1.02	0.31	0.30	7.79
Pvul-EIL-2	Glyma.05G180300	1.02	0.31	0.31	7.80
Pvul-EIL-2	Glyma.11G239000	0.93	0.16	0.17	7.19
Pvul-EIL-2	Glyma.18G018400	0.71	0.16	0.23	5.44
Pvul-EIL-3	Glyma.05G180300	0.31	0.14	0.44	2.33
Pvul-EIL-3	Glyma.11G239000	1.65	0.27	0.17	12.71
Pvul-EIL-3	Glyma.18G018400	1.61	0.33	0.20	12.45
Pvul-EIL-4	Glyma.06G068400	1.83	0.32	0.17	14.14
Pvul-EIL-4	Glyma.04G066900	1.89	0.32	0.16	14.58
Pvul-EIL-4	Glyma.13G166200	0.27	0.05	0.19	2.07
Pvul-EIL-4	Glyma.17G113900	0.24	0.05	0.21	1.86
Pvul-EIL-5	Glyma.06G314000	0.78	0.28	0.36	6.02
Pvul-EIL-6	Glyma.13G076700	0.48	0.09	0.19	3.69
Pvul-EIL-6	Glyma.20G051500	0.60	0.09	0.15	4.65
Pvul-EIL-8	Glyma.14G041500	0.48	0.06	0.13	3.72
Pvul-EIL-8	Glyma.20G051500	1.09	0.14	0.13	8.45
Pvul-EIL-10	Glyma.06G068400	0.28	0.1	0.35	2.17
Pvul-EIL-10	Glyma.04G066900	0.27	0.09	0.34	2.14
Pvul-EIL-10	Glyma.14G116800	0.67	0.20	0.30	5.17
Pvul-EIL-10	Glyma.17G113900	2.23	0.35	0.15	17.21
Pvul-EIL-10	Glyma.17G211000	0.74	0.22	0.29	5.75

Table 4. Orthologous relationships identification of EIL genes among the P. vulgaris, G. max and A. thaliana genomes

ID	Gene ID	Ks	Ka	Ka/Ks	MYA
Pvul-EIL-1	AT5G25350	3.51	0.31	0.09	27.02
Pvul-EIL-1	AT2G25490	1.97	0.27	0.13	15.15
Pvul-EIL-10	AT5G25350	4.18	0.38	0.09	32.21
Pvul-EIL-10	AT2G25490	2.53	0.34	0.13	19.53

Results and Discussion

Identification, Chromosomal Localization, and Duplication Analysis of EIL Genes in P. vulgaris

In this study, 11 *EIL* genes/proteins were identified and these *EIL* genes were named from *Pvul-EIL-1* to *Pvul-EIL-11* (Table 1). The number of *EIL* genes in *P. vulgaris* was found to be more than *Arabidopsis thaliana* (n = 6) (Chao et al., 1997), *Prunus mume* (n = 5), *Prunus persica* (n = 4), *Fragaria vesca* (n = 5) (Cao et al., 2017), *Zea mays* (n = 9) (Jyoti et al., 2021), but less than the number of members identified in *Gossypium hirsutum* (n = 18) (Salih et al., 2020), *Glycine max* (n = 12) (Li et al., 2019) and *Triticum aestivum* (n = 21) (Yi-Qin et al., 2020).

EIL proteins identified in *P. vulgaris* were determined to be between 447 and 651 amino acids in length and between 51.08 and 70.68 kDa in molecular weights. In addition, Pvul-EIL-1, Pvul-EIL-9, and Pvul-EIL-10 proteins were found to have basic properties, while the remaining Pvul-EIL members were found to have acidic properties (Table 1). Similarly, in a study conducted on cotton plants, it was determined that the members mostly had acidic properties (Salih et al., 2020). According to GRAVY values varying between 0.186 and -0.845, Pvul-EIL-1, -4, -9, and -10 of Pvul-EIL proteins were found to be hydrophobic, while other members were found to be hydrophilic (Table 1). Subcellular localizations of Pvul-EIL members were defined using the WoLF PSORT: Protein subcellular localization prediction tool (Horton et al., 2006). Based on this analysis, all Pvul-EILs were predicted to be localized in the nucleus (Table 1). Similarly, EIL proteins from *G. max* (Li et al., 2019), *T. aestivum* (Yi-Qin et al., 2020), *P. trichocarpa* (Filiz et al., 2017), and *Z. mays* (Jyoti et al., 2021) species have also been reported to be localized in the nucleus. Instability

index values of all Pvul-EIL members except Pvul-EIL-1 were found to be above 40. This showed that all members of the identified Pvul-EIL proteins, except Pvul-EIL-1, were unstable. The aliphatic index can be regarded a positive factor for increasing global protein thermostability, in addition to being the relative volume filled by aliphatic side chains (alanine, valine, isoleucine, and leucine) (Ikai 1980). The aliphatic index values of the Pvul-EIL proteins ranged between 63.01 and 109.92, indicating the strong thermostability of the proteins (Table 1).

Pvul-EIL genes were found to be localized in 7 of 11 chromosomes of *P. vulgaris* (Chr 1, -2, -3, 5, -6, -8, -9). *Pvul-EIL-1* and *-2* were situated on chromosome 1, *Pvul-EIL-6* and *-7* were placed on chromosome 6, *Pvul-EIL-9* and *-10* were located on chromosome 9. On the other hand, *Pvul-EIL-3*, *-4*, *-5*, and *-8* genes were located on chromosomes 2, 3, 5, and 8 respectively (Figure 1). Also, the *Pvul-EIL-11* gene was paired as an unknown chromosome (scaffold). Similarly, scaffold was determined for one of the *EIL* genes identified in *P. mume* (Cao et al., 2017) and *G. hirsutum* (Salih et al., 2020). In this study, the *Pvul-EIL* genes were shown to be unevenly distributed in the chromosomes of *P. vulgaris*, similar to previous studies on diverse plant species (Filiz et al., 2017; Jyoti et al., 2021; Li et al., 2019).

Gene duplications are the production of gene copies that can cause the expansion, development and evolution of gene families (Büyük et al., 2021a). This study was conducted to analyze the evolutionary status of duplication and divergence of *Pvul-EIL* genes (Table 2). Homologous (Ks), non-homologous (Ka), and Ka/Ks values were calculated to gather more information regarding the evolution of duplicating genes. Ka/Ks>1 means positive selection or Darwinian selection, Ka/Ks<1 means purifying or negative selection, and Ka=Ks means neutral selection or no selection (Zhang et al., 2006). As a result, segmental duplication gene pairs were identified in 5 of 11 *Pvul-EIL* genes. The Ka/Ks ratio of duplicated *EIL* genes in the genome of *P. vulgaris* varied between 0.01 and 0.26. In other words, it was determined that the duplicated *Pvul-EIL* genes (Ka/Ks<1) were under purifier selection pressure. Similarly, in studies conducted in *Glycine max* (Li et al., 2019) and *Populus trichocarpa* (Filiz et al., 2017), it was reported that duplicated *EIL* genes were also found as Ka/Ks<1, and they were under purifying selection pressure.

Orthologous genes are genes found in different animals that share a same evolutionary origin, resulting in structural and functional similarities, but diverged throughout the species formation process (Miller et al., 2019). *EIL* genes determined to be orthologous between *P. vulgaris* - *G. max* species were given in Table 3. A total of 26 gene pairs were orthologous and the genes showing the most orthologous relationship between *P. vulgaris* - *G. max* species were *Pvul-EIL-1* and *Pvul-EIL-10*. According to the analysis performed between *Pvul-EIL* and *GmEIL*, orthologs were mostly observed in chromosome 1. According to the findings, Ks values ranged from 0.24 to 2.23, Ka/Ks values from 0.13 to 0.44, and MYA values from 1.86 to 17.2. Moreover, the mean values of Ks, Ka/Ks, and MYA were found to be 0.89, 0.23, and 6.84, respectively (Table 3).

Table 4 listed the *EIL* gene pairs that were discovered to be orthologous between the *P. vulgaris* and *A. thaliana* species. A total of 4 gene pairs were found to show orthologous relationship. The genes showing orthologous relationship from *Pvul-EIL* genes were *Pvul-EIL-1* and *Pvul-EIL-10*. Their Ks values varied between 1.97 and 4.18, Ka/Ks values between 0.09 and 0.13, and MYA values between 15.15 and 32.21 (Table 4). The detected orthologous relationships demonstrated structural and functional similarities between the *EIL* genes of *P. vulgaris*, *A. thaliana*, and *G. max*.

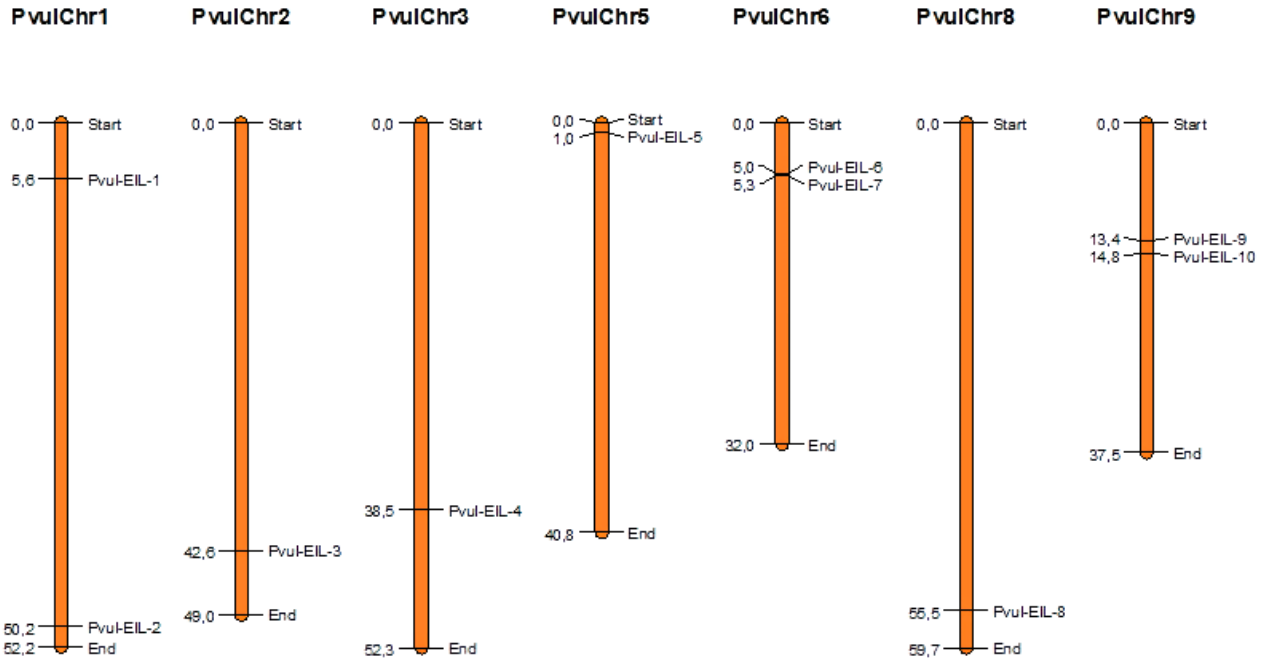

Figure 1. Distribution of *Pvul-EIL* genes on *P. vulgaris* chromosome

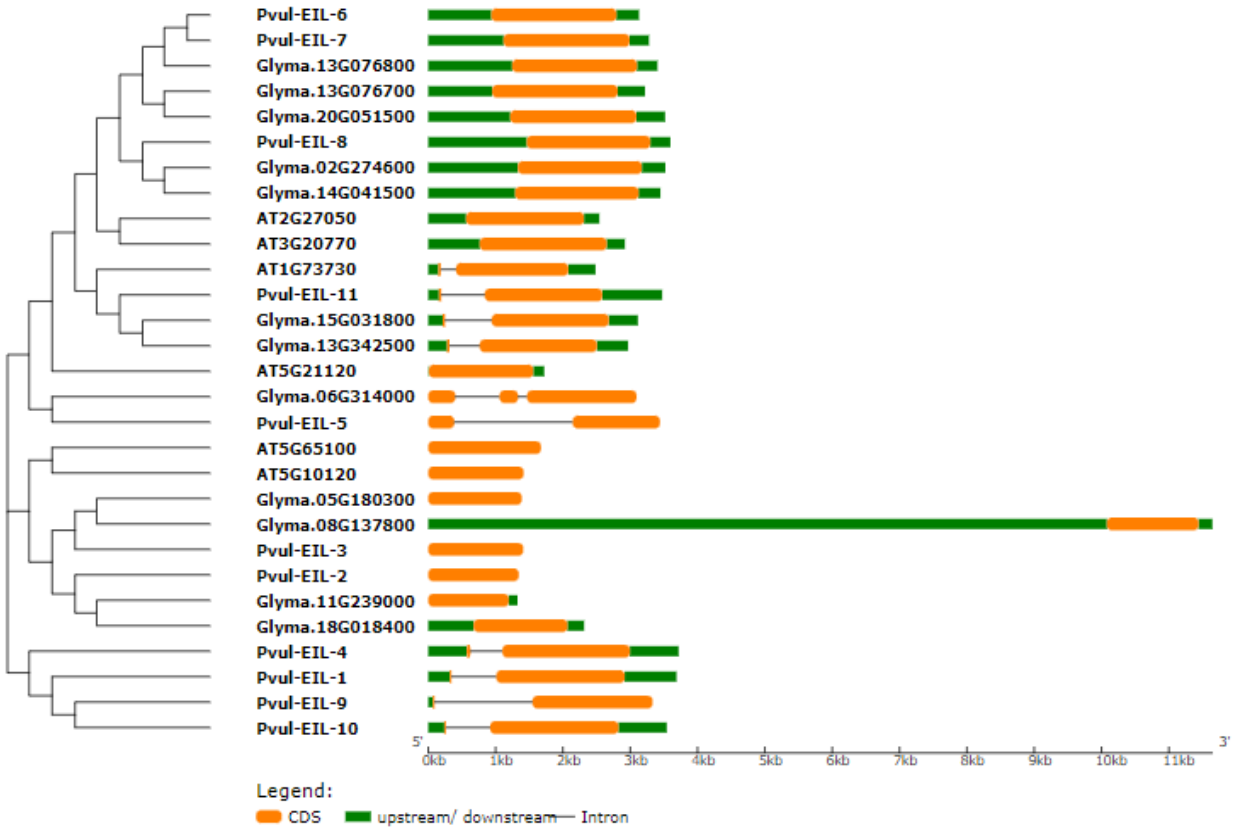

Figure 2. Gene structures of *EIL* family members from *P. vulgaris*, *A. thaliana and G. max* by clustering based on NJ-based phylogenetic tree. Introns are showed by lines.

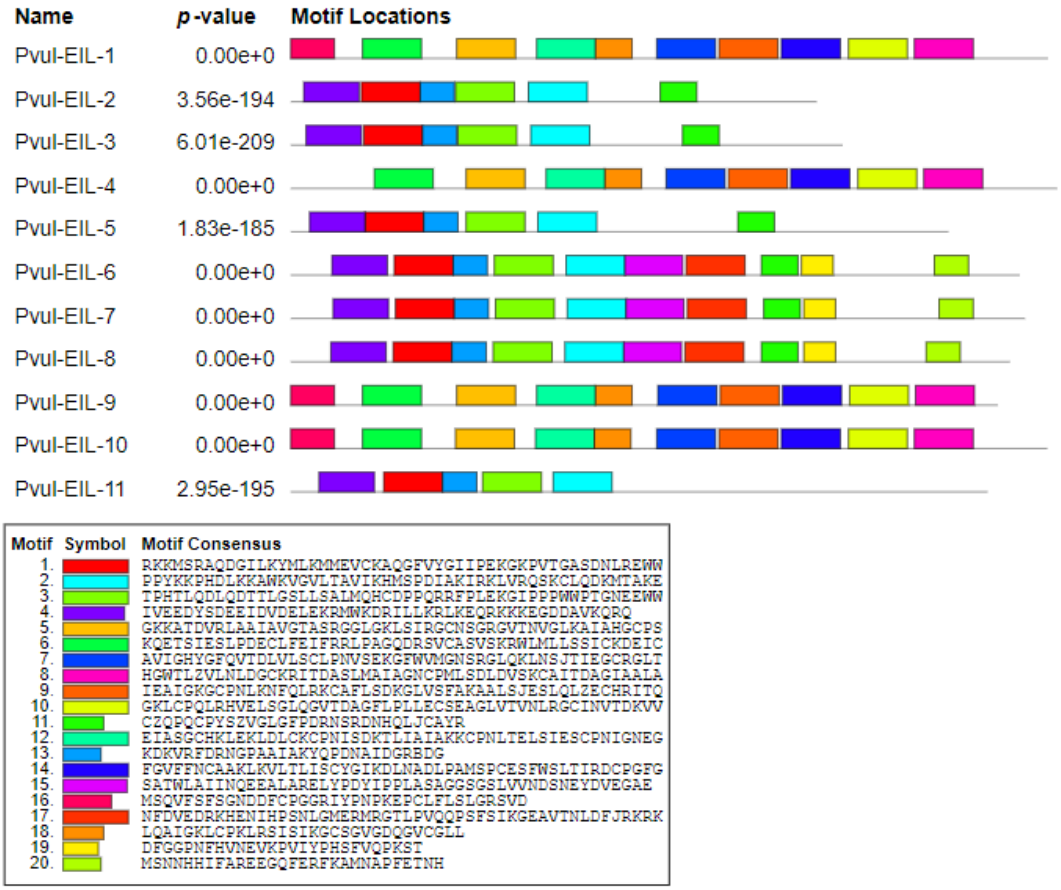

Figure 3. Conserved-motifs of Pvul-EIL proteins of *P. vulgaris*. Pvul-EIL proteins have 20 conserved motifs, as shown in this diagram. The motifs were obtained using the MEME online program.

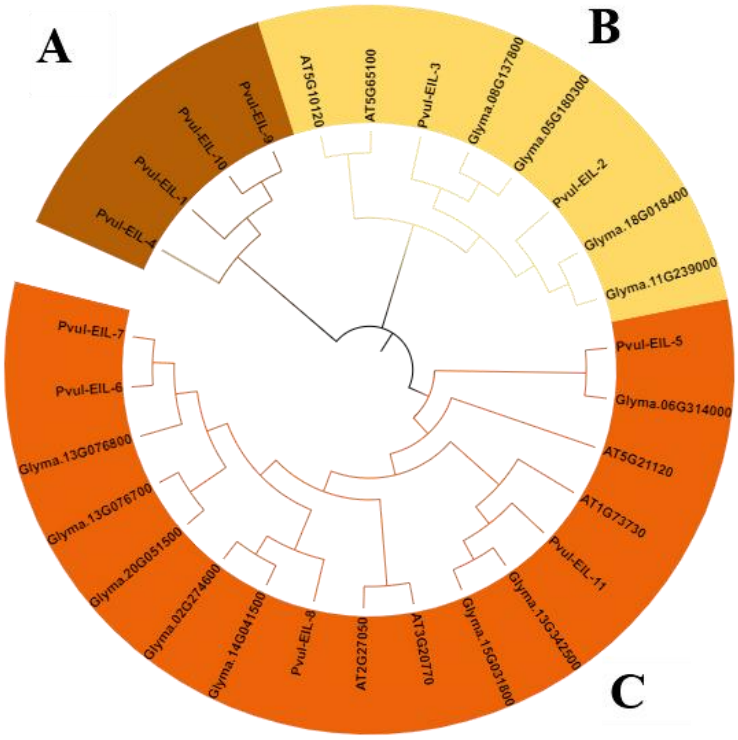

Figure 4. The NJ method was applied to construct the phylogenetic tree. In, *Arabidopsis thaliana*, *Glycine max* and *Phaseolus vulgaris*, the descriptive names of EIL proteins begin with 'Pvul', 'AT', and 'Glyma', respectively.

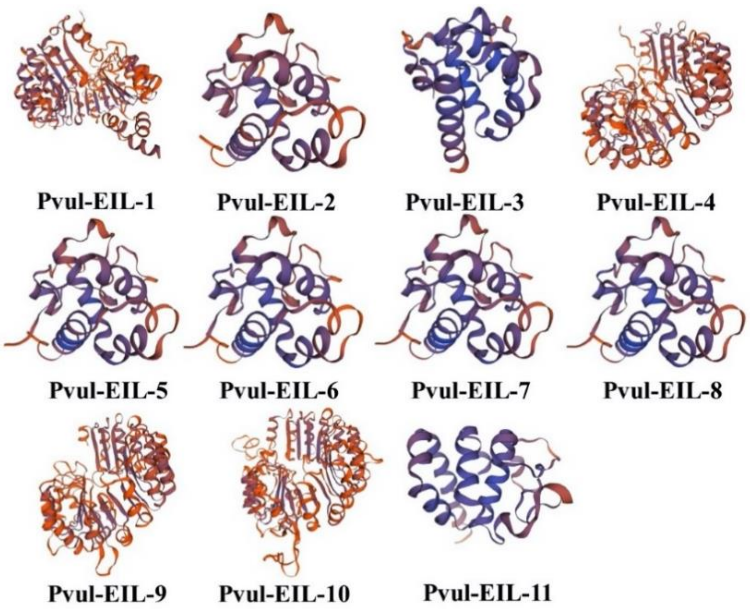

Figure 5. 3D structures of Pvul-EIL proteins

Gene Structure, Motif Analysis, Homology Modeling, and Phylogenetic Analysis of EIL Members in P. vulgaris

The physical structure of the *Pvul-EIL* genes identified in the *P. vulgaris* genome was analyzed (Figure 2). It was determined that while *Pvul-EIL-1, -4, -5, -9, -10,* and *-11* genes contained one intron per gene, the remaining *Pvul-EIL-2, -3, -6, -7,* and *-8* genes did not contain introns. Similarly, in a study conducted by Salih et al., (2020) on cotton species, it was determined that some *EIL* genes did not contain introns (Salih et al., 2020). In other studies, it was observed that *EIL* genes mostly include exons (Cao et al., 2017; Jyoti et al., 2021; Salih et al., 2020). Furthermore, the *EIL* gene structures of *G. max* and *A. thaliana* were examined, and it was shown that the majority of *EIL* genes only had exons (Figure 2). Obtained exon-intron profiles contributed to the understanding of gene structure, motifs, and phylogenetic relationships of *Pvul-EIL* genes.

Moreover, motif compositions of Pvul-EILs were investigated. Accordingly, 20 different conserved Pvul-EIL protein motifs, amino acid sequences, and motif lengths were given in Figure 3. Motifs are short regions of protein sequences that are conserved, mutation-resistant, and exhibit evolutionary and functional distinctions (Mangan and Alon 2003). According to the analyses, Pvul-

EIL-1, Pvul-EIL-9, and Pvul-EIL-10, Pvul-EIL-2 and Pvul-EIL-3, Pvul-EIL-6, Pvul-EIL-7, and Pvul-EIL-8 were found to have the same motif compositions (Figure 3). Pvul-EIL proteins with the same motif compositions were also found under the same clade in the phylogenetic tree. The validity of the phylogenetic analysis and the gene structure profiles were evaluated in light of these findings (Figure 3).

As the result of phylogenetic analyses conducted for detecting the evolutionary relationship between Pvul-EIL proteins and EIL proteins from the *A. thaliana* and *G. max* genomes, three different groups were emerged (n_A=4, n_B=8, and n_C=17) (Figure 4). Of the segmental duplicated gene pairs, Pvul-EIL-1 and Pvul-EIL-10, Pvul-EIL-2 and Pvul-EIL-3, Pvul-EIL-4 and Pvul-EIL-10, Pvul-EIL-6 and Pvul-EIL-8 were classified under the same groups in the phylogenetic tree. The motif content of Pvul-EIL-6 and Pvul-EIL-7 has been proven to be identical, and they were located under the same node in group C in the phylogenetic tree. Furthermore, Pvul-EIL-8, which was highly similar to these two proteins, had the same motif content, and belonged to phylogenetic group C. Similar to our findings, in the study conducted by Cao et al. (2017) it was determined that PbEIL9 and PbEIL10 proteins both contained the same motif compositions, and were in the same group in the phylogenetic tree (Cao et al., 2017).

Three-dimensional (3D) structure prediction was performed using homology modeling for 11 Pvul-EIL proteins (Figure 5). Accordingly, EIL proteins mainly mapped to three main structures. One of which is the three-dimensional structure of the AtEIN3 (SMTL ID: 4zds.1) protein's DNA binding domain (DBD) that consists of six -helixes and five short helices (Song et al., 2015). The other one is the three-dimensional structure of AtEIL3's DNA binding domain (SMTL ID: 1wij.1) which consists of five α-helixes (Yamasaki et al., 2005). Another one is the structure with less than 20% similarity in sequences (SMTL ID: 2p1m.1.B).

While the three-dimensional models derived from the peptide sequences of Pvul-EIL-1, Pvul-EIL-4, Pvul-EIL-9, and Pvul-EIL-10 had a reliability level of less than 20%, the three-dimensional models derived from the peptide sequences of other members had a reliability level of more than 60%.

Cis-acting elements are critical molecular regulators that control the transcription of genes that governs a number of biological processes such abiotic hormone responses, stress (Wittkopp and Kalay 2012). In this study, promoter analysis was undertaken in order to identify cis-regulatory elements of *Pvul-EIL* genes. The identified elements were divided into following 8 main groups: environmental stress, biotic stress, hormone, growth, light, promoter binding site and others (Table S1). *Pvul-EIL-6*, *Pvul-EIL-7* and *Pvul-EIL-8* genes had the most cis-regulatory elements and were grouped under the same group in the phylogenetic tree.

TATA-box and CAAT-box, main promoter elements, were found in all *Pvul-EIL* genes as expected. These promoters play role in hormonal signals, the regulation of gene expression, control of growth and development, and translation into appropriate morphological or physiological responses (Li et al., 2019). Detailed promoter analysis showed that *Pvul-EIL* genes, except for the *Pvul-EIL-11* gene, had different hormone-related elements such as auxin, gibberellin, abscisic acid, ethylene, methyl jasmonate, and salicylic acid (Table S1). *Pvul-EIL* genes with hormone-related elements from most to less can be put in order as *Pvul-EIL-6* (10), *Pvul-EIL-7* (9), *Pvul-EIL-2*, and *Pvul-EIL-9* (6), *Pvul-EIL-8* and *Pvul-EIL-10* (5), *Pvul-EIL-4* (4), *Pvul-EIL-5* (3), *Pvul-EIL-1* (2), *Pvul-EIL-3* (1). The gene with the largest number of hormone-related elements was *Pvul-EIL-6*. In addition, genes with the auxin element were *Pvul-EIL-6* and *Pvul-EIL-10*. Moreover, the fact that the *Pvul-EIL-6* and *Pvul-EIL-7* genes encode proteins with identical hormone-related regions and the same motif composition implies that the analyses were in agreement (Figure 3). According to these findings, *EIL* genes play a significant role in phytohormone signaling pathways and can be controlled transcriptionally by a variety of hormones.

Detection of miRNAs Targeting the Pvul-EIL Genes

miRNAs can be defined as a part of non-coding RNAs that are 22 nucleotides in length, and play a role in regulating various aspects of plant growth and development (Salih et al., 2020, Sun 2012). According to the studies, miRNAs are believed to have a role in plant growth and development, as well as the plant's response to abiotic and biotic stress factors (Wu et al., 2017). As a result of the miRNA analysis performed in this study, a total of 423 *Pvul-EIL* related miRNAs were identified (Table S2). The gene targeted by the largest number of miRNAs was *Pvul-EIL-8*. When all *Pvul-EIL* genes were considered, it was discovered that miRNAs targeting the most *Pvul-EIL* genes belonged to the miR167, miR399, and miR172 families. miRNAs targeting Pvul-EIL members the most (miR167, miR399, miR169, miR393, miR397, miR156, miR1861) were regarded as hormone-related (ethylene, abscisic acid, gibberellin, auxin hormone, etc.) activities in the literature. According to the results of Pramoolkit et al., (2014) study on *Hevea brasiliensis* plant, genes targeted by miR167 may play a role in the ethylene response (Pramoolkit et al., 2014). Song et al., (2015) in another study on ethylene and plant response in phosphate deficiency, it was determined that ethylene, which plays a role in regulating both regional and systemic signals, was associated with miR399 (Song and Liu 2015). Chung et al., (2020) studied ethylene development at five fruit development stages by gas chromatography to determine the role of miR172 in fruit ethylene production. As a result of the aforementioned study, it was stated that overexpression of miR172 increased ethylene biosynthesis, fruit color, and additional ripening properties, especially by suppressing SlAP2a (Chung et al., 2020). As a result, it was understood that miR167, miR399, and miR172, which are known to be associated with ethylene responses, target *Pvul-EIL* genes more in number than other miRNAs (Chung et al., 2020; Pramoolkit et al., 2014; Song et al., 2014) (Table S2).

In a study conducted by Song et al., (2018), it was understood that miR169 family, which is known to have a role in biotic and abiotic stress factors, plays an important role in the regulation of the ABA pathway (Song et al., 2018). *Pvul-EIL-6*, *Pvul-EIL-7*, and *Pvul-EIL-8* genes targeted by miR169 were also found to contain ABA hormone-related cis elements (Table S1).

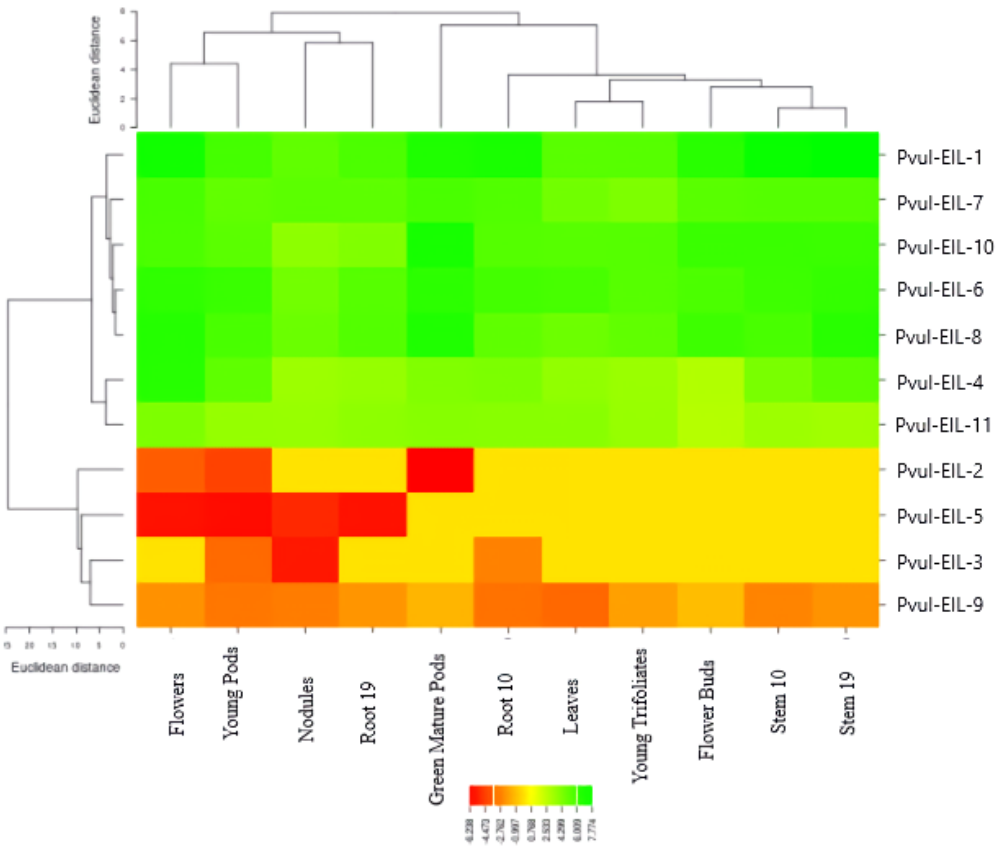

Figure 6. Tempo-spatial expression patterns of *Pvul-EIL* genes in different organs of *P. vulgaris*. Green and red on the color scale show high and low transcript expression, respectively.

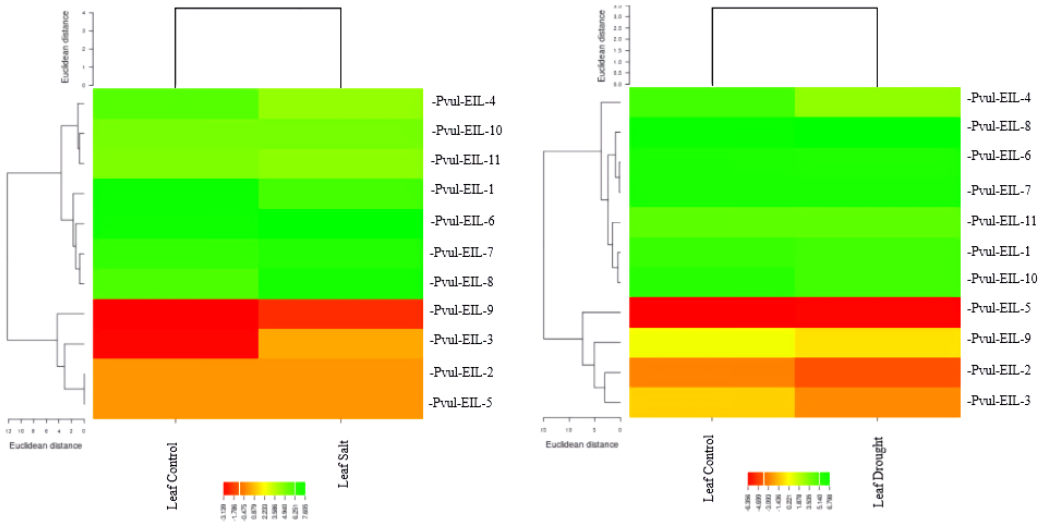

Figure 7. Heatmap of *Pvul-EIL* genes achieved by RNAseq studies which shows differentially expressed genes under control/salt stress and control/drought stress conditions.

miRNA families that play a role in abiotic and biotic stress factors, as well as hormone-related miRNAs, were identified as a result of miRNA study on Pvul-EIL members (Table S2). It was determined by Huang et al., that miR397 has important roles in plant embryonic development, flower organ, and seed development, fruit development, flowering as well as some abiotic stress factors (Huang et al., 2020). In the Wang et al., (2019)'s study, miR156 were considered as the factor which is responsible for plant growth and salt stress tolerance (Wang et al., 2019). Finally, in a miRNA study conducted by Ai et al., (2021) on *Oryza sativa*, it was determined that miR1861 participates in the control of plant growth, development, and response to abiotic stresses, and is a positive regulator of plant tolerance to salt stress (Ai et al., 2021).

Tissue-specific mRNA Levels of the Pvul-EIL Genes

The plotted heat-map showed varying levels of expression of the *Pvul-EIL* genes 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 6). In most of the analyzed tissues, *Pvul-EIL-1*, *-4*, *-6*, *-7*, *-8*, *-10*, and *-11* genes were found to

be expressed at much higher levels than other genes. It is noteworthy that the *Pvul-EIL-6, -7,* and *-8* genes, which have orthologous association with *G. max*, had high and similar gene expression levels in most of the analyzed tissues (Table 3). On the other hand, being on the same chromosome, having the same motif compositions, and being in the same phylogenetic group (group C) were suggested to be the explanation for high and similar expression levels of *Pvul-EIL-6* and *Pvul-EIL-7* genes in the analyzed tissues (Figs. 1, 3, 4). In contrast, the *Pvul-EIL-2, Pvul-EIL-3, Pvul-EIL-5,* and *Pvul-EIL-9* genes were expressed at low levels in the analyzed tissues (Figure 6).

Responses of Pvul-EIL Genes to Salt and Drought Stress Through RNAseq Analysis

RNAseq analysis of *Pvul-EIL* genes was performed using SRA data developed by Hiz et al., (2014) (Büyük et al., 2019; Büyük et al., 2021c; Hiz et al., 2014; Yıldız et al., 2021). As a result, when compared to the control, certain genes were expressed at lower levels, and others at higher levels under salt and drought stress conditions (Figure 7). It was determined that the *Pvul-EIL-1, -4, -6, -7, -8, -10,* and *-11* genes showed high expression levels under both stress and control conditions in both heat maps (Figure 7). *Pvul-EIL-2, Pvul-EIL-3, Pvul-EIL-5 and Pvul-EIL-9* genes were determined as low-expressed genes according to both heat maps, and all of these genes seemed to respond to at least one of the salt or stress conditions (Figure 7). In addition, it was determined that the *Pvul-EIL-2, Pvul-EIL-3, Pvul-EIL-5* and *Pvul-EIL-9* genes, which were found to show low expression in Figure 7, also showed low expression in the plant tissues presented in Figure 6.

Conclusion

In conclusion, this study provides a comprehensive genome-wide analysis of the *EIL* genes in *P. vulgaris*. A total of 11 detected *EIL* genes were named from *Pvul-EIL-1* to *Pvul-EIL-11*. Various analyses were performed by the help of bioinformatics tools and genome databases to gain insight into the biological roles of *EIL* genes in the *P. vulgaris* genome. Chromosomal location, duplication, ortholog, and gene structure analysis of the determined gene family, homology of proteins, conserved motif analysis, phylogenetic analysis, analysis of promoter regions, miRNA analysis, tissue-specific mRNA, and determination of salt and drought stress levels were performed. The results of this work, in which *EIL* genes were identified and described for the first time in *P. vulgaris*, are regarded to be a valuable resource for future research on *EIL* genes in *P. vulgaris* and other plant species.

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