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The NADPH Oxidases (*NOX*) Gene Family Expression and Genome-Wide Characterization in Common Beans (*Phaseolus vulgaris* L.)

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Abstract: This study aimed to identify and characterize the NADPH oxidases (*NOX*) gene family in the common bean (*Phaseolus vulgaris* L.) to understand its role in plant growth, development, and stress responses. Using bioinformatic tools, the *NOX* gene family members were identified and analyzed for their molecular weights, isoelectric points, amino acid numbers, and evolutionary relationships. Segmental duplication analysis and phylogenetic studies were conducted using *NOX* genes from *Arabidopsis thaliana*, *Cicer arietinum*, *Oryza sativa*, and *Glycine max*. The results revealed nine Phvul-NOX proteins in the common bean genome, with molecular weights ranging from 92940.09 to 105660.24 kDa, isoelectric points from 7.86 to 9.36, and amino acid numbers from 823 to 946. Segmental duplication was observed in *Phvul-NOX-1*/*Phvul-NOX-3*, *Phvul-NOX-2*/*Phvul-NOX-8*, and *Phvul-NOX-5*/*Phvul-NOX-6* gene pairs, and purifying selection was identified throughout the evolutionary process. Phylogenetic analysis grouped the *NOX* genes into three main clades, and a synteny map between *A. thaliana* and *P. vulgaris* was constructed. This study provides the first comprehensive characterization of the *NOX* gene family in the common bean, offering valuable insights for future functional genomics research and potential applications in enhancing stress tolerance and crop productivity.

Keywords: Abiotic stress, bioinformatic, common bean, functional genomics, phylogenetic, reactive oxygen species

1. Introduction

As signaling and harmful chemicals, reactive oxygen species (ROS) have a multifaceted function in biological systems. The crucial impacts of ROS on the integrity, health, and aging of organisms are reflected in this biological conundrum (D'Autréaux and Toledano, 2007; Chang et al., 2016). Because of their cell membrane-bound architecture, NADPH oxidases (NOX), the primary enzymes responsible for the formation of ROS, are essential to many biological processes in both plants and animals (Torres and Dangl, 2005; Bedard et al., 2007; Bedard and Krause, 2007). NOX enzymes function as a "hub" in signaling pathways controlled by ROS. NOX enzymes, often referred to as "respiratory burst oxidases" (RBOH), were initially discovered in human phagocytes and have since been investigated in a variety of taxa, including fungi, mammals, and plants (Bedard et al., 2007; Chang et al., 2016; Zhang et al., 2019). Animals have been shown to contain seven different forms of NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. There are many NOX members in various species, even if only NOX5-like NOX kinds are present in plants (Sagi and Fluhr, 2006; Bedard et al., 2007; Bedard and Krause, 2007; Wang et al., 2013; Hu et al., 2018). NOXs typically take part in a number of processes, including absisic acid (ABA)-mediated stomatal closure (Zhang et al., 2009; Shi et al., 2012), apoptosis (Tewari et al., 2012), tapetal cell death, pollen development (Xie et al., 2014), polar growth of root hairs (Nestler et al., 2014), growth in pollen tubes (Kaya et al., 2014, 2015; Wudick and Feijó, 2014), and plant immune response (Yoshioka et al., 2011). Numerous plant

categories, such as lower plants, dicotyledons, and monocotyledons, have been shown to have more than 150 NOX family proteins (Hu et al., 2020). As the gp91phox homolog in mammals, the first plant NOX protein was identified from *Oryza sativa* (rice) plants (Groom et al., 1996). Since then, NOXs have been found in a wide variety of plants, including maize (*Zea mays*) (Nestler et al., 2014; Chang et al., 2016), tomato (*Lycopersicon esculentum*) (Amicucci et al., 1999), tobacco (*Nicotiana tabacum*) (Yoshioka et al., 2001), potato (*Solanum tuberosum*) (Yoshioka et al., 2003), *Arabidopsis thaliana* (Sagi and Fluhr, 2006), wheat (*Triticum aestivum*) (Hu et al., 2018), and *Medicago truncatula* (Marino et al., 2011). The possible roles of several NOX proteins in various plant species in ROS generation in plant stress responses are still being thoroughly examined, despite the fact that some members of the NOX family have been well examined (Ye et al., 2024).

A food crop in the Fabaceae family, *P. vulgaris* is a member of the legume family and is significant to the global economy (Silva-Gigante et al., 2023; Cheng et al., 2024). Environmental stressors including drought, salt, and cold have an impact on bean output (Wang et al., 2024). Abiotic stressors have a detrimental effect on the growth and development processes of plants, leading to worldwide yield losses. These stressors have detrimental impacts on plant growth and can lead to oxidative damage in plant tissues, which can result in structural and functional abnormalities (Hasanuzzaman et al., 2013). Plants have evolved extremely intricate defensive systems that have been preserved throughout evolution to deal with harsh environmental circumstances. Among these conserved systems, the ROS signaling network plays important roles in plant growth and stress responses (Hasanuzzaman et al., 2013; Wang et al., 2024). In higher plants, *NOX* genes have a variety of vital roles in sustaining normal growth and adapting to abiotic and biotic stressors (Zhang et al., 2019). There are, however, little research on the functional characterization of the bean's *NOX* genes. The objective of this study was to employ in silico methods to characterize the entire genome of the *NOX* gene family members that regulate the bean's response to abiotic stress. Through the analysis of gene architectures, chromosomal distribution, duplication patterns, and expression profiles of *NOX* genes in beans, we aimed to identify potential candidate genes in this study. This research presents an extensive examination of the *NOX* gene family within the common bean, yielding significant insights that may inform future endeavors in functional genomics and hold promise for improving stress resilience and agricultural yield.

2. Materials and Methods

2.1. Identification of *Phvul-NOX* genes

Members of the *Phvul-NOX* gene family were found using the Pfam database's accession numbers (PF08414). Protein, transcript, genomic, and CDS sequences were obtained from the Phytozome v13 database (https://phytozome-next.jgi.doe.gov/). Potential NOX proteins were found in the genomes of *Arabidopsis thaliana* (Lamesch et al., 2011), *Cicer arietinum*, *Oryza sativa*, and *Glycine max* (Valliyodan et al., 2019) using the Phytozome v13 Hidden Markov Model (HMM) (http://www.ebi.ac.uk) and the BLASTp tool. Furthermore, the HMMER database (http://www.ebi.ac.uk) verified the Phvul-NOX domain's existence. The Expasy ProtParam program (https://web.expasy.org/protparam) was used to examine the theoretical isoelectric point (pI), molecular weight, instability index, and amino acid number of bean NOX proteins. The WoLF SPORT database was used to forecast their subcellular localizations (Horton et al., 2007).

2.2. Exon/Intron structure analysis and chromosomal location of *Phvul-NOX* genes

Intron and exon regions of Phvul-NOX proteins were analyzed from the GSDS (Gene Structure Display Server v2.0) database using genomic and CDS (CoDing Sequences) sequences (Guo et al., 2007). Phytozome v13 was used to establish the chromosomal locations and sizes of *Phvul-NOX* genes, and the "TBtools" tool was used to display the genes chromosomal positions (Chen et al., 2023). Tbtools computed evolutionary strains (Ka/Ks), synonymous ratios (Ks), and non-synonymous ratios (Ka) between gene pairs (Chen et al., 2023).

2.3. Identification of conserved motifs and promoter analysis

The "MEME Suite" was used to identify *Phvul-NOX* genes for analysis of conserved motifs (Bailey et al., 2006); the maximum motif number was 10 and the motif width was restricted to 6-50 (Aygören et al., 2022). The InterProScan database was used to examine the motifs that were collected (Quevillon et al., 2005). The Phytozome database v13 was used to obtain the 2000 bp upstream regions of the *Phvul-NOX* genes, and cis-acting elements were found using the PlantCARE database. After that, the TBtools were to generate the phenogram (Lescot et al., 2002; Chen et al., 2023).

2.4. Phylogenetic analysis, synteny analysis, and 3D homology modeling

MEGA v11 was used for multiple sequence alignment and phylogenetic analysis (Tamura et al., 2021). The iTOL program was used to show the phylogenetic tree, which was created using the repeated neighbor-joining method using 1000 bootstrap values (Thompson et al., 1997; Letunic and Bork, 2011; Kasapoğlu et al., 2024). The "One Step MCScanX" program was used to compare the genome and transcript sequences of *A. thaliana* and *P. vulgaris*, and the "TBtools" software was used to show the synteny relationships between these two species (Chen et al., 2023). The sequences of Phvul-NOX proteins were used to obtain 3D structures using Phyre2 (Kelley et al., 2015). With the help of the Chimera interface, the best 3D image of the protein models was to be visualized (Pettersen et al., 2004).

2.5. Homology modeling and protein-protein interactions (PPI) of Phvul-NOX proteins

The 3D structures of Phvul-NOX proteins were determined using Phyre2 (Kelley et al., 2015). Protein-protein interactions both functional and physical characteristics were obtained from the STRING database (https://stringdb.org/). These interaction data were categorized and presented using Cytoscape software (Shannon et al., 2003).

2.6. Gene expression analysis (in-silico)

Phvul-NOX genes were studied using Illumina RNA-seq data from the NCBI (National Center for Biotechnology Information) Sequence Reading Archive (SRA) database. Accession numbers SRR957668 (leaf under salt stress), SRR958469 (leaf under salt control) (Hiz et al., 2014), SRR8284480 (leaf under drought control), and SRR8284481 (leaf under drought stress) were used to get the appropriate RNA-seq data. Log2 transformation of RPKM (Reads per kilobase: Transcripts per kilobase) values was used to calculate in silico expression profiles (Mortazavi et al., 2008), and Tbtools was used to generate a heatmap (Chen et al., 2023).

3. Results and Discussion

NOX genes belonging to *P. vulgaris*, *A. thaliana*, *C. arietinum*, *O. sativa*, and *G. max* species were identified by the Pfam number (PF08414) in the Pfam database and keyword searches in the Phytozome v13 browser. According to homology analyses, 17 *NOX* genes were identified in *G. max* (Zhang et al., 2019), 10 in *A. thaliana* (Chang et al., 2016), 9 in *O. sativa* (Chang et al., 2016) and 36 in *T. aestivum* (Hu et al., 2018), while 9 *NOX* genes were identified in this study on the bean. The amino acid number of Phvul-NOX proteins varied from 823 to 946. The amino acid numbers of Phvul-NOX proteins ranged from 823 to 946. The gene with the highest amino acid number was found to be Phvul-NOX-3, while the gene with the lowest amino acid number was found to be Phvul-NOX-4. The molecular weights of Phvul-NOX proteins ranged from 92,940.09 to 105,660.24 kDa; Phvul-NOX-1 had the highest molecular weight, and Phvul-NOX-4 had the lowest molecular weight. When the theoretical isoelectric point was evaluated, the lowest value was 7.86 (Phvul-NOX-2) and the highest value was 9.36 (Phvul-NOX-4).

It was determined that *Phvul-NOX* genes were distributed on the 6 chromosomes of the bean, namely PhvulChr02, PhvulChr03, PhvulChr06, PhvulChr07, PhvulChr08 and PhvulChr09. Among these genes, the chromosome with the highest number of *Phvul-NOX* genes (*Phvul-NOX-1*, *Phvul-NOX-2*, and *Phvul-NOX-3*) was determined as Chr02. Comparatively, in the wheat genome, *NOX* genes distribution was observed on Chr1, Chr3, Chr4, Chr5, and Chr6 (Hu et al., 2018). In addition, gene duplication analyses in the bean genome revealed three segmental duplicated gene pairs (*Phvul-NOX-1*/*Phvul-NOX-3*, *Phvul-NOX-2*/*Phvul-NOX-8*, and *Phvul-NOX-5*/*Phvul-NOX-6*) (Figure 1). No genes with tandem duplications

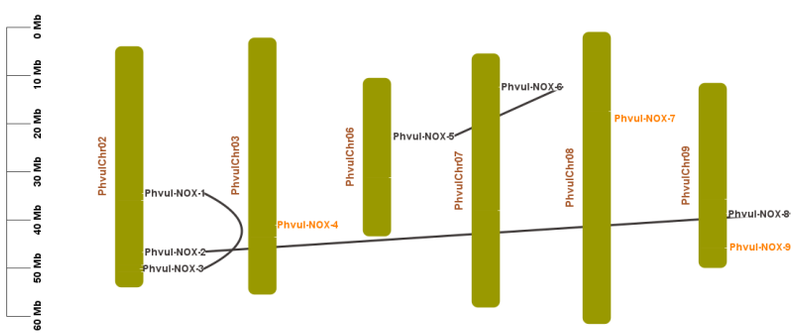

Figure 1. Distribution of the chromosomal locations of *Phvul-NOX* genes*

*: Shown in black indicates segmental duplication.

were found in this analysis. A total of eight segmental duplicated gene pairs were detected in the *GmNOX* gene family, which suggests the gene family's expansion could have been caused by whole genome duplication (WGD) or segmental duplications (Zhang et al., 2019). It is also suggested that these segmental duplications observed in both the bean and the soybean may have played an important role in the *NOX* gene family's evolutionarily expanding. In addition, to understand the evolutionary dynamics of duplicated genes in more detail, the evolutionary processes of segmental and tandem duplications of *NOX* genes were investigated by calculating the Ka and Ks change rates and their ratio (Ka/Ks) (Table 1). The Ka/Ks ratio for bean NOX genes ranged from 0.10 to 0.23. Ka/Ks>1 value indicates positive selection while Ka/Ks<1 indicates purifying selection, and Ka/Ks= 1 indicates neutral selection (Li et al., 2009; Khan et al., 2020; Buttanri et al., 2024). The results show that duplicated *NOX* genes in the bean have undergone purifying selection, and similarly, the *GmNOX* gene family's Ka/Ks ratios in the soybean (Zhang et al., 2019).

Table 1. The evolutionary status of duplicated *Phvul-NOX gene* pairs in *P. vulgaris*

Gene 1	Chr	Gene 2	Chr	Ka	Ks	Ka/Ks	Selective pressure	Duplicate type
Phvul-NOX-1	2	*Phvul-NOX-3*	2	0.08	0.69	0.12	purifying selection	segmental
Phvul-NOX-2	2	*Phvul-NOX-8*	9	0.16	0.70	0.23	purifying selection	segmental
Phvul-NOX-5	6	*Phvul-NOX-6*	7	0.08	0.79	0.10	purifying selection	segmental

Xu et al. (2012), Kasapoğlu et al. (2020), and Buttanri et al. (2024) have found that gene duplication contributes to evolution through intron/exon gain or loss, exonization, and deletion/insertion. While intron positions in orthologous genes are highly conserved over long evolutionary periods, the exon/intron structure may be less conserved; however, introns in paralogous genes can remain stable enough to allow the examination of evolutionary relationships (Rogozin et al., 2003; Li et al., 2009). Both exon and intron numbers and positions of the *Phvul-NOX* genes were determined by comparing CDS and genomic DNA sequences (Figure 2). *Phvul-NOX* genes exon numbers ranged from 12 to 14, with *Phvul-NOX-2* and *Phvul-NOX-8* having the lowest intron-exon numbers. It was found that these two genes had similar exon/intron structures and that most of the *NOX* genes generally exhibited similar exon/intron structures. In addition, in *A. thaliana* and *O. sativa* studies, it was determined that *NOX* genes were classified into four conserved subfamilies as I, II, III, and IV based on their exon/intron organization; Subfamilies I contained 6-14 introns, while the variation was 11-13, 11-14, and 7-17 introns within the subfamily groups of II, III, and IV, accordingly (Chang et al., 2016). The WoLF PSORT database analysis revealed that the majority of *NOX* genes are situated in the plasma membrane and nucleus. Furthermore, it was shown that these genes are situated in several intracellular areas, including mitochondria, cytoplasm, and endoplasmic reticulum (Table 2). While all *Phvul-NOX* genes in the bean were discovered to be situated in the plasma membrane, all *NOX* genes in wheat were anticipated to be situated within the cell plasma membrane based on Plant-mPLoc predictions. Although conventional transmembrane helices were not found in *TaNOX* counterparts, it was expected that these proteins would be located in the plasma membrane (Hu et al., 2018).

The Phvul-NOX protein contains typical regions of plant NOXs. The ferric reductase-like transmembrane component (Ferri_reductase domain), the FAD-binding domain (FAD-binding_8 domain), and the ferric reductase NAD-

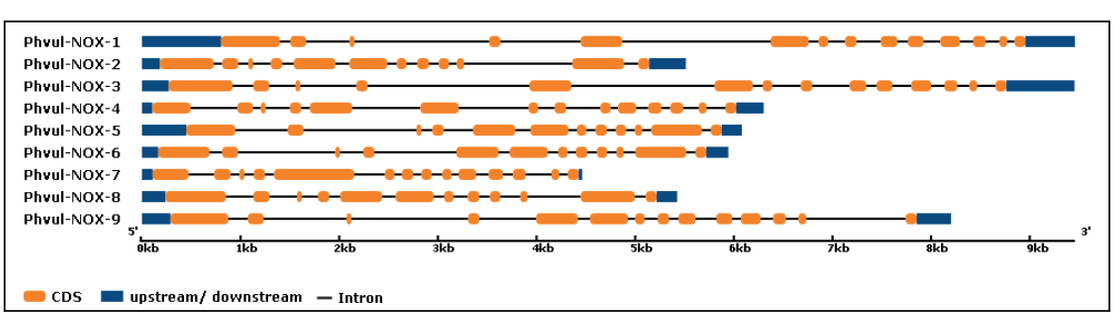

Figure 2. Number, length, and position of exons and introns in the *Phvul-NOX* genes*

*: Orange boxes indicate 5-UTR and 3-UTR regions, dark blue boxes indicate exons, and black lines indicate intron regions.

Table 2. Cellular locations of the Phvul-NOX genes

Gene ID	Cellular location
Phvul-NOX-1	plas: 10, nucl: 2, chlo: 1, E.R.: 1
Phvul-NOX-2	plas: 13, E.R.: 1
Phvul-NOX-3	plas: 10, nucl: 2, E.R.: 1, pero: 1
Phvul-NOX-4	plas: 10, nucl: 3, mito: 1
Phvul-NOX-5	plas: 12, nucl: 2
Phvul-NOX-6	plas: 13, nucl: 1
Phvul-NOX-7	plas: 5, E.R.: 4, vacu: 2, nucl: 1, extr: 1, golg: 1
Phvul-NOX-8	plas: 12, nucl: 1, E.R.: 1
Phvul-NOX-9	plas: 5.5, E.R.: 5, cyto_plas: 3.5, nucl: 1, mito: 1, pero: 1

chlo: Chloroplast, cyto: Cytosol, nucl: Nucleus, mito: Mitochondria, plas: Plasma membrane, cysk: Cytoskeleton, extr: Extracellular, per: Peroxisome, vacu: Vacuole, E.R: Endoplasmic reticulum, golg: Golgi

binding domain (NAD-binding_6 domain) are included in these regions. Moreover, more similar characteristics were found in these conserved functional regions. The NADPH_Ox domain is necessary for NOXs and is in charge of the production of ROS. Similar typical regions have been found in other plants (rice, wheat, soybean, and *A. thaliana*), and the EF-hand motif has been identified in all NOX proteins. These major regions are distributed in similar locations and sizes in various NOX sequences (Chang et al., 2016; Hu et al., 2018; Zhang et al., 2019). Both the structural and functional predictions of NOX proteins as well as their 3D homology modeling are conveyed in a visually understandable manner (Figure 3).

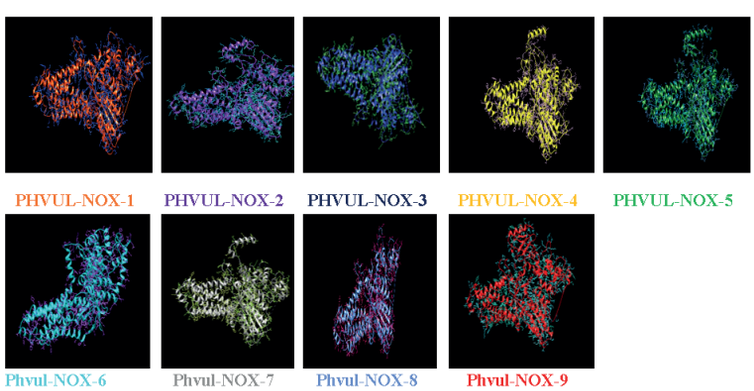

Figure 3. 3D modeling of the Phvul-NOX proteins

The promoter regions of *NOX* genes contain a variety of cis-acting elements. Figure 4 displays the 26 most common cis-acting elements. The focus is on elements that respond to environmental stress and hormones. It has been determined that these elements, especially myeloblastosis (MYB), G-box, salicylic acid responsive elements (TCA-element), and absisic asid responsive element (ABRE), regulate plant development, plant hormones, and abiotic stress responses (Hu et al., 2018; Ye et al., 2024). These cis-acting elements help regulate gene expression, enabling plant adaptation under stress conditions. In particular, the promoter regions of *Phvul-NOX* genes support molecular responses to environmental stresses. In addition, *GmNOX* genes were up-regulated to

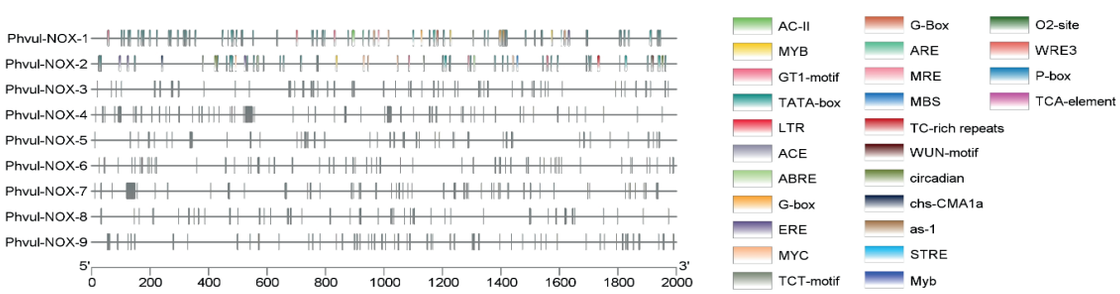

Figure 4. Cis-acting element analysis of the *Phvul-NOX* genes

phytohormones such as abscisic acid (ABA), jasmonate (JA), and salicylic acid (SA) indicating that NOX genes play important roles in stress responses associated with these hormones (Zhang et al., 2019). The promoter regions of NOX genes contain elements like ABRE, which have essential roles in improving plant tolerance against abiotic stresses (Ye et al., 2024). The study in *Opisthopappus taihangensis* identified elements such as CAAT-box related to phytohormones and abiotic stress (Ye et al., 2024), observing ROS signaling pathways are associated with phytohormone regulatory pathways; to examplify, ABA may improve tomato stress tolerance by increasing the expression of *NOX* gene (Zhou et al., 2014), while JA regulates the antioxidant enzyme system and NOX activity (Alam et al., 2014; Ye et al., 2024). Abiotic stresses including salinity, drought, and high temperature can have a detrimental effect on the growth and development of plants. Plant cells recognize those stresses and use ROS signaling to produce adaptive responses; regulatory mechanisms of ROS production are crucial for those stress responses (Jaspers and Kangasjärvi, 2010; Suzuki et al., 2011).

Phvul-NOX gene expression under drought and salt stress was investigated in in-silico analyses on common beans. The expression of *Phvul-NOX-1*, *Phvul-NOX-6*, and *Phvul-NOX-7* genes increased under salt stress, while the expression of other genes decreased in comparison to the control, according to the evaluation conducted using RNAseq data (SRR957668, SRR958469, SRR8284480, and SRR8284481) retrieved from the NCBI SRA database. While other genes showed changes as a result of salt and drought stress, *Phvul-NOX-6* gene expression specifically increased under drought stress (Figure 5).

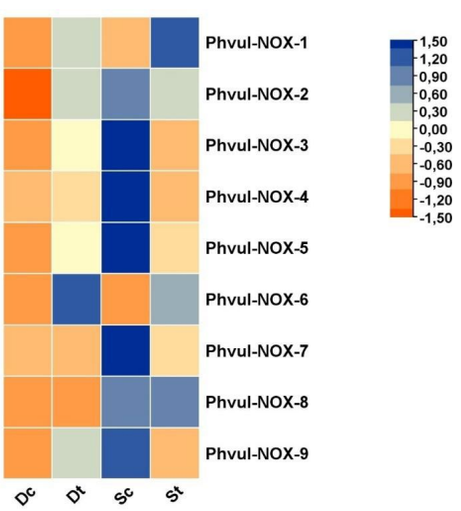

Figure 5. Heatmap representation of the *Phvul-NOX* gene

In salt-control conditions, increased expression was observed especially in *Phvul-NOX-3*, *Phvul-NOX-4*, and *Phvul-NOX-5* genes. Furthermore, in *O. taihangensis*, up-regulation under drought stress and down-regulation under salt stress were noted (Ye et al., 2024), and four *NOX* gene candidates were found to be up regulated under drought stress in soybean analyses (Zhang et al., 2019). Important indications about gene function can be identified via patterns of gene expression (Du et al., 2012).

4. Conclusions

This study revealed that *Phvul-NOX* genes were distributed to 6 chromosomes and 9 *Phvul-NOX* genes were obtained through an in-silico examination of the *NOX* gene family found in the bean. In addition, it was determined that all Phvul-NOX proteins have NADPH_Ox domain and include some typical regions. It was observed that NOXs in plasma membranes play an important role in stress conditions as well as ROS production and plant development processes. This study not only sheds light on the *NOX* genes functions in the bean but also provides a guide for many future studies.

Ethical Statement

The author declares that ethical approval is not required for this research.

Funding

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Declaration of Conflicts of Interest

No conflict of interest has been declared by the author.

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