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GENOME-WIDE IDENTIFICATION AND CHARACTERIZATION OF *NODULE INCEPTION-LIKE PROTEIN* (*NLP*) GENE FAMILY IN MUNGBEAN (*VIGNA RADIATA* L.)

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SUMMARY

NODULE-INCEPTION (NIN)-like proteins (NLPs) are critical in regulating nitrogen (N) use in plants. Although *NLPs* are well-studied in various species, their characterization in mungbean (*Vigna radiata* L.) remains limited. The identified *VrNLPs* totaling seven shared similarities in their physicochemical properties with *Arabidopsis thaliana NLPs* (*AtNLPs*). A comparison of conserved domains confirmed that *VrNLPs*, like *AtNLPs*, contain both the RWP-RK and PB1 domains, verifying their membership in the same gene family. Analysis of gene structures revealed similar exon-intron patterns between *VrNLPs* and *AtNLPs*; although, *VrNLPs* had shorter gene lengths. In contrast, the average protein lengths of *VrNLPs* showed higher similarity to those of *AtNLPs*. Both sets of proteins appeared to be hydrophilic, as indicated by the negative Grand Average of Hydropathicity (GRAVY) values. Subcellular localization analysis indicated that all *VrNLPs* are nuclear-localized. Overall, *VrNLPs* and *AtNLPs* share substantial homology in gene structure, protein domains, motifs, and physicochemical attributes. The phylogenetic analysis exhibited *VrNLPs* and *AtNLPs* as closest relatives, suggesting their evolution from a common ancestor alongside *NLPs* from other vascular and non-vascular plants (*Oryza sativa*, *Zea mays*, and *Physcomitrella patens*). An improved nitrogen use efficiency (NUE) could lead to higher yields with reduced fertilizer input, mitigating environmental pollution from excessive fertilizer use.

Keywords: mungbean, nitrogen, nitrogen use efficiency, Nodule Inception-like proteins, transcription factor

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Key findings: This study identified members of the *NLP* gene family in mungbean (*Vigna radiata* L.) and provided a preliminary functional overview of *VrNLPs*. With their structural and functional similarities with *AtNLPs*, *VrNLPs* showed promising potential for enhancing NUE in mungbeans through molecular plant breeding and genetic engineering.

INTRODUCTION

Nitrogen (N) is an essential element comprising 78% of the Earth's atmosphere and playing a vital role in plant structure, function, growth, and yield (Wang *et al.,* 2024). Given their critical importance, nitrogenous fertilizers' common application in agriculture helps enhance crop productivity. However, despite its atmospheric abundance and extensive agricultural use, crops typically utilize only 30%–40% of applied N, with the remaining 60%–70% being lost into the soil system (Li *et al.,* 2017). This inefficiency contributes to environmental pollution and reduces farmers' economic returns (Anas *et al.,* 2020). Consequently, improving N use efficiency (NUE) through both crop breeding programs and agronomic practices has become a crucial agricultural objective. Major factors responsible for low N uptake include volatilization, surface runoff, leaching, and innate plant potential to use N (Govindasamy *et al.,* 2023).

One of the most effective ways to enhance plant NUE is the identification and subsequent genetic engineering of key genes and transcription factors (TFs) involved in the plant's N uptake, transport, assimilation, utilization, and overall NUE (Alfatih *et al.,* 2020; Alfatih *et al.,* 2023). The identification of several critical TFs involved in N signaling has succeeded, which regulate genes associated with N uptake, assimilation, and related metabolic pathways (Nazish *et al.,* 2022). Among these, the NODULE-INCEPTION-LIKE or NIN-like protein (NLP) TF family, initially discovered for its role in legume nodulation, plays a critical role in N metabolism and nitrate signaling in plants (Xiao *et al.,* 2020; Sámano *et al.,* 2024). The NIN protein serves as the founding member of the NLP family and is distinctive for its RWP-RK and PB1 domains. RWP-RK is a DNA-binding domain characterized by a highly conserved pattern of arginine-tryptophan-proline (RWP) and arginine-lysine (RK), allowing its protein to bind to specific DNA sequences in target gene promoters and regulate the expression of genes. Meanwhile, the presence of the PB1 domain classifies legume NIN proteins as NLPs. Notably, while NIN proteins are restricted to legumes, NLPs have been evident in diverse non-leguminous species of rice, *Arabidopsis*, wheat, and maize (Samano *et al.,* 2024).

Given the importance of improving NUE in crop plants and the demonstrated role of NLP-TFs in enhancing NUE, it is crucial to confirm the presence of NLPs in target species before genetic modification approaches. Although *NLPs* have been characterized in many crops, but investigating their presence and functions in mungbean remains untapped. Therefore, this study sought to comprehensively identify *NLP* family members in the mungbean genome, characterize their structural features and functional potential for NUE improvement, and assess the evolutionary relationship of *VrNLPs* with economically important crop species.

MATERIALS AND METHODS

Screening of genome and transcription factors' databases

The complete gene, amino acid, and coding sequences for each member of the *AtNLP* gene family came from The *Arabidopsis* Information Resource (TAIR: http://arabidopsis.org). A total of three protein databases, including the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/), Phytozome v13 (https://phytozome-next.jgi.doe.gov/), and Plant TF Database (PlantTFDB: http://planttfdb.gao-lab.org/) became choices to screen for putative *VrNLPs,* using either *AtNLPs* protein sequences or accession numbers of the domains, such as PF02042 (RWP-RK) and PF00564 (PB1). All

retrieved sequences incurred downloading in the FASTA format and archiving for subsequent sampling and analysis.

Removal of redundant sequences

The aligning and filtering of all *VrNLP* protein sequences ensured the removal of redundant, spliced, and incomplete sequences. Duplicate sequences—identified through both sequence similarity analysis and matching accession numbers across databases—reached systematic elimination. Sequences from NCBI, Phytozome, and PlantTFDB, upon aligning, matched identical sequences from different databases to ensure data integrity by preventing duplication while maintaining specificity and accuracy.

Conserved domain confirmation and physicochemical properties

The putative *VrNLPs* selected after redundancy removal further sustained verification using NCBI's Conserved Domain Database (CDD) to confirm the presence of characteristic domains. Only proteins containing both RWP-RK and PB1 domains succeeded in retention as candidate *VrNLPs*, while sequences possessing either one or none of these domains bore elimination. Physical and chemical properties, including molecular weight (MW), grand average of hydropathy/hydropathicity (GRAVY), and isoelectric point (pI), underwent analysis using ProtParam on ExPASy (https://web.expasy.org/protparam/) (Gasteiger *et al.,* 2003). Subcellular localization predictions, as performed used CELLO (http://cello.life.nctu.edu.tw/) (Yu *et al.,* 2014). Both the physicochemical properties and subcellular location of *VrNLPs* attained comparison with *AtNLPs*.

Motif composition and gene structure analysis

Consensus motifs' identification engaged the Multiple EM for Motif Elicitation (MEME) suite version 5.1 (https://meme-suite.org/meme/tools/meme), a well-validated tool for motif discovery (Bailey *et al.,* 2015). All parameters remained at default values, except for the motif threshold set to 15 to optimize detection specificity. These conserved motifs reveal functionally important regions shared between the reference species and our study organism.

For structural characterization, both coding and genomic sequences of *VrNLPs* acquired analysis using the Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn/). This analysis precisely mapped intron-exon boundaries and untranscribed regions (UTRs), while also enabling comparative assessment of gene lengths across different gene families (Hu *et al.,* 2015).

Phylogenetic analysis

Protein sequences of NLPs from *Arabidopsis thaliana*, *Zea mays*, *Physcomitrella patens*, *Oryza sativa*, and *Vigna radiata* were used for multiple sequence alignment using Clustal Omega online (https://www.ebi.ac.uk/Tools/msa/clustalo/). The aligned sequences then served to construct a maximum likelihood phylogenetic tree with MEGA11 (Molecular Evolutionary Genetics Analysis version 11). Finally, applying the Interactive Tree of Life (iTOL) platform (https://itol.embl.de/) helped process the Newick format tree file for visualization.

RESULTS

Screening mungbean genome indicated presence of *VrNLPs*

The amino acid sequences of all *Arabidopsis* NLP members (AtNLP1-9) served as queries for BLASTp searches against the NCBI proteome database. Matching sequences continued retrieval in FASTA format, with their accession numbers and percentage similarity recorded. Since *Vigna radiata's* proteome is not available in Phytozome v13, exclusion of this database transpired from our analysis. From PlantTFDB, all sequences listed under the NLP category in FASTA format succeeded in retrieving. Following self-alignment of the retrieved

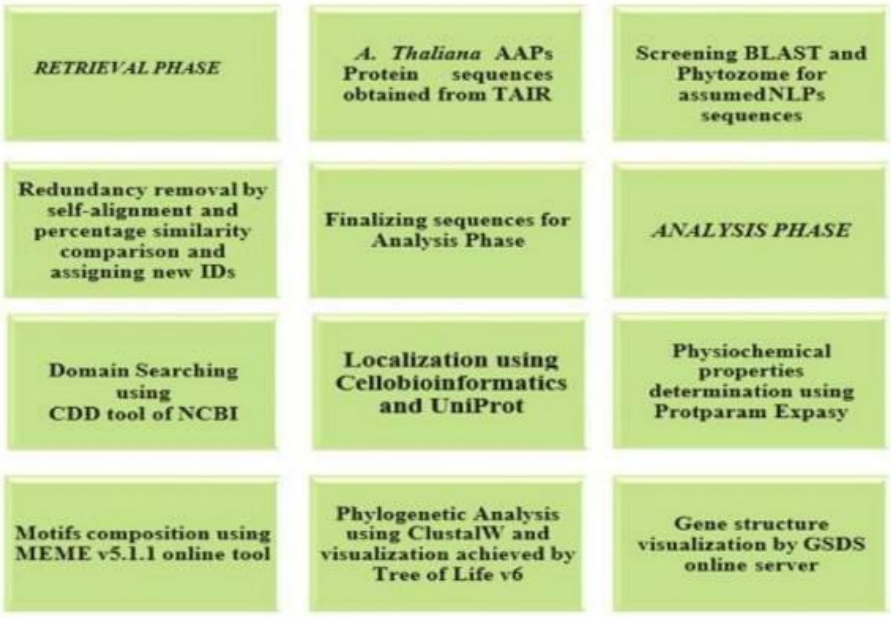

Figure 1. Workflow diagram.

Table 1. List of *VrNLPs* with their NCBI accession numbers after redundancy removal and confirmation through CDD.

No.	Accession Numbers	Given Names
1	XP_014502050.1	*VrNLP*1
2	XP_022636040.1	*VrNLP*2
3	XP_022637353.1	*VrNLP*3
4	Vradi08g18730.1	*VrNLP*4
5	Vradi01g14220.1	*VrNLP*5
6	XP_014494469.1	*VrNLP*6
7	XP_014490160.1	*VrNLP*7

sequences from both databases and removal of redundant entries based on their accession numbers, ultimately, 12 non-redundant sequences as putative *VrNLPs* achieved being retained. The detailed methodology appears in Figure 1.

AtNLPs and *VrNLPs* share similarities in their domains and physicochemical properties

From the initial 12 putative *VrNLPs*, CDD analysis confirmed that only seven sequences contained both RWP-RK and PB1 domains while the remaining five sequences either contained one or no domain. The selected seven *VrNLPs*, listed in Table 1 with their NCBI accession numbers, received the designation as *VrNLP*1-7 according to their chromosomal order. Table 2 provides a comprehensive comparison of domains between *VrNLPs* and *AtNLPs*, including query details, hit type, position, e-value, bit score, and accession numbers. Both *VrNLP* and AtNLP proteins exhibited highly conserved RWP-RK and PB1 domains, providing preliminary confirmation that the selected sequences represent genuine *VrNLPs*.

Table 2. Features of conserved domains identified in *AtNLPs* and *VrNLPs.*

	Query	Hit type	Position		E-Value	Bit-score	Accession Number	Short Name
			From	To				
Arabidopsis thaliana	*AtNLP1*	Specific	812	893	6.54E-41	112.802	cd06407	PB1_NLP
			608	656	2.62E-24	54.0518	pfam02042	RWP-RK
	AtNLP2	Specific	896	944	1.26E-41	112.802	cd06407	PB1_NLP
			648	696	4.74E-24	54.0518	pfam02042	RWP-RK
	AtNLP3	Specific	674	758	1.56E-40	112.802	cd06407	PB1_NLP
			498	546	1.69E-24	54.0518	pfam02042	RWP-RK
	AtNLP4	Specific	745	826	7.15E-43	112.802	cd06407	PB1_NLP
			558	606	1.67E-24	54.0518	pfam02042	RWP-RK
	AtNLP5	Specific	711	787	3.72E-36	112.802	cd06407	PB1_NLP
			549	597	5.58E-25	54.0518	pfam02042	RWP-RK
	AtNLP6	Specific	742	822	3.01E-34	112.802	cd06407	PB1_NLP
			556	604	8.48E-25	54.0518	pfam02042	RWP-RK
	AtNLP7	Specific	866	944	4.32E-34	112.802	cd06407	PB1_NLP
			591	639	4.66E-25	54.0518	pfam02042	RWP-RK
	AtNLP8	Specific	848	928	6.25E-39	112.802	cd06407	PB1_NLP
			590	651	1.25E-20	54.0518	pfam02042	RWP-RK
	AtNLP9	Specific	793	874	3.37E-34	112.802	cd06407	PB1_NLP
			535	584	5.08E-25	54.0518	pfam02042	RWP-RK
Vigna radiata	*VrNLP1*	Specific	827	907	1.42E-42	112.802	cd06407	PB1_NLP
			607	655	7.44E-24	54.0518	pfam02042	RWP-RK
	VrNLP2	Specific	685	766	3.76E-40	112.802	cd06407	PB1_NLP
			493	541	1.48E-23	54.0518	pfam02042	RWP-RK
	VrNLP3	Specific	648	729	6.18E-37	112.802	cd06407	PB1_NLP
			514	562	2.16E-23	54.0518	pfam02042	RWP-RK
	VrNLP4	Specific	945	1025	1.07E-38	112.802	cd06407	PB1_NLP
			635	683	8.88E-25	54.0518	pfam02042	RWP-RK
	VrNLP5	Specific	903	983	1.98E-36	112.802	cd06407	PB1_NLP
			595	643	4.56E-24	54.0518	pfam02042	RWP-RK
	VrNLP6	Specific	867	947	1.54E-29	112.802	cd06407	PB1_NLP
			598	646	2.38E-24	54.0518	pfam02042	RWP-RK
	VrNLP7	Specific	879	959	6.83E-31	112.802	cd06407	PB1_NLP
			602	650	4.16E-24	54.0518	pfam02042	RWP-RK

Analysis and comparison of physicochemical properties of both *VrNLPs* and *AtNLPs* showed similar protein characteristics for gene length, protein length, molecular weight, pI, and GRAVY values. The negative GRAVY values observed in both plants suggest hydrophilic protein properties. Subcellular localization predictions indicated nuclear localization for *NLPs* in both species. The average protein lengths were 881 and 916 amino acids for *AtNLPs* and *VrNLPs*, respectively. Meanwhile, protein properties were largely similar, with a notable variation in gene lengths between the two species observed. All *NLPs* from both species exhibited acidic properties (pI < 7), except for *AtNLP3,* which showed basic characteristics (Table 3).

***AtNLPs* and *VrNLPs* share resemblance in their gene structure and motif patterns**

Although *VrNLPs* lacked UTRs (Figure 2), their exon-intron patterns displayed similarities in both number and structure with *AtNLPs*. Among the seven *VrNLPs*, *VrNLP*2 and *VrNLP*3 were the shortest genes, while *VrNLP*7 was the longest among all examined NLPs from both species. Comparative motif analysis revealed that 10 out of 15 motifs (75%) were shared between *AtNLPs* and *VrNLPs* (Figure 3). The

Table 3. Physical and chemical properties of *NLP* gene families of *Arabidopsis thaliana* and *Vigna radiata.*

	Gene Name	Chromo-some	Position	Gene length (bp)	Protein length (aa)	Mol. weight	Iso-electric Point	GRAVY	Localization
Arabidopsis thaliana	*AtNLP1*	1	22324437-2327359	2933	909	100886.25	5.09	-0.443	Nuclear
	AtNLP2	4	16777264-6782054	4791	963	107278.54	5.65	-0.476	Nuclear
	AtNLP3	4	17954710-7958063	3354	767	85066.66	8.35	-0.271	Nuclear
	AtNLP4	4	7154410-7158287	3878	844	94231.93	5.45	-0.472	Nuclear
	AtNLP5	5	17367827-7369510	1684	808	90684.13	5.8	-0.467	Nuclear
	AtNLP6	4	950546-953690	3145	841	93863.46	5.95	-0.356	Nuclear
	AtNLP7	5	1698270-1702659	4390	959	105742.04	5.55	-0.42	Nuclear
	AtNLP8	2	18061716-8066708	4993	947	104883.79	5.47	-0.43	Nuclear
	AtNLP9	3	22009008-2012804	3797	894	98712.97	5.43	-0.383	Nuclear
Vigna radiata	*VrNLP1*	5	23422183-3427061	4879	920	102669.81	5.67	-0.48	Nuclear
	VrNLP2	5	24677902-4680551	2650	783	87014.05	5.99	-0.371	Nuclear
	VrNLP3	6	94900-97518	2619	731	81509.27	5.93	-0.351	Nuclear
	VrNLP4	8	40521825-0526233	4409	963	106382.02	5.33	-0.458	Nuclear
	VrNLP5	1	34948877-4954133	5257	979	108365.47	5.84	-0.351	Nuclear
	VrNLP6	2	3664175-3671010	6836	1002	111677.29	5.82	-0.409	Nuclear
	VrNLP7	Un	12240-20459	8220	1039	115185.97	5.61	-0.323	Nuclear

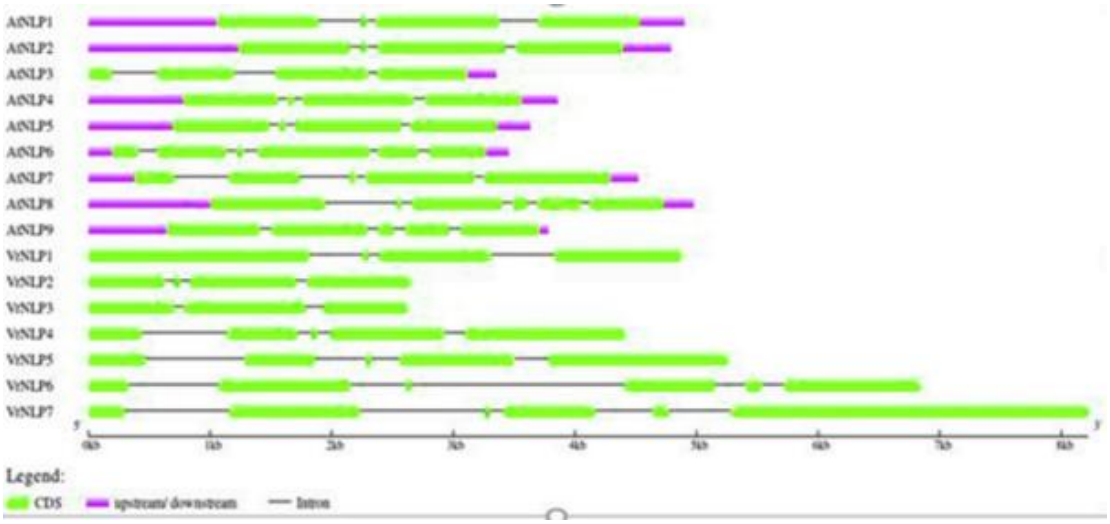

Figure 2. Gene structure features of *AtNLPs* and *VrNLPs.*

high conservation observed in both gene structure (nucleotide sequences) and motif patterns (protein sequences) further confirms the accurate identification of *VrNLPs*.

AtNLPs and *VrNLPs* are the closest orthologs

Phylogenetic analysis elucidated the evolutionary relationships between *VrNLPs* and *NLP* gene families from other species. The *VrNLPs* displayed distinct divergence patterns across the phylogenetic tree, revealing their evolutionary connections with five representative plant species (Figure 4). Comparative analysis showed that *VrNLPs* share the closest structural and functional similarities with *AtNLPs* and *ZmNLPs*. The phylogenetic reconstruction supports a common ancestral origin for all *NLP* genes,

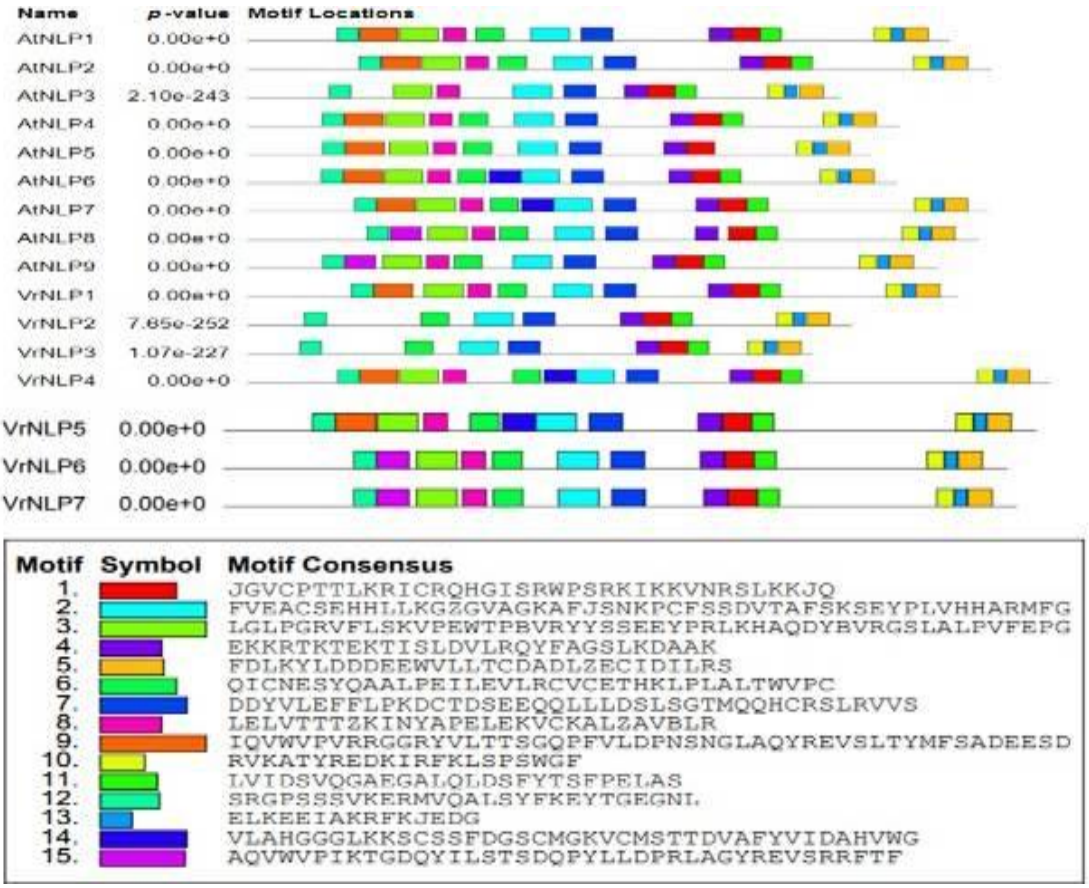

Figure 3. Consensus motifs of *AtNLPs* and *VrNLPs.*

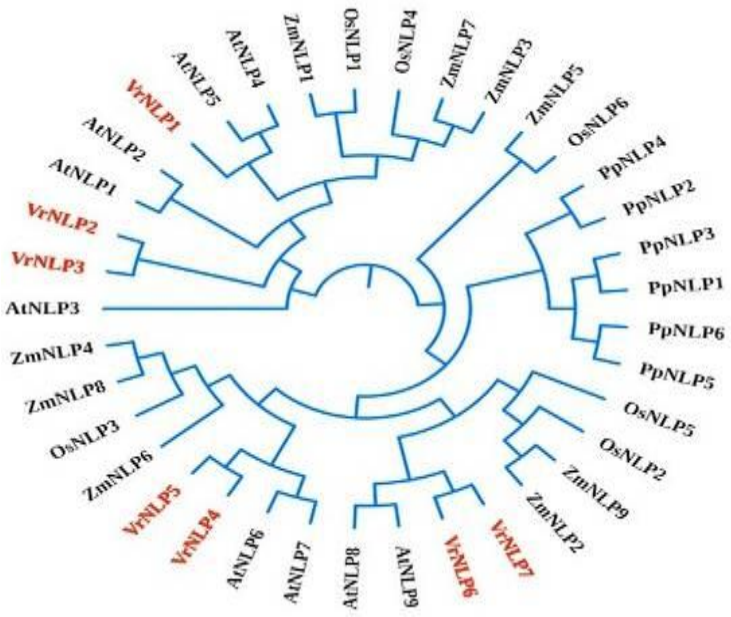

Figure 4. Phylogenetic analysis of *VrNLPs* and other plant species *NLPs*.

with subsequent divergence into distinct gene families. Within the *VrNLP* family, *VrNLP6* and *VrNLP7* demonstrated closer evolutionary relationships than *VrNLP4* and *VrNLP5*. The tree topology exhibited two primary clades, each bifurcating into two subclades that further separated into smaller groups, mirroring their structural and functional relationships. Notably, *PpNLPs* formed an isolated clade, consistent with their non-vascular plant origin. Furthermore, *AtNLP3* clustered separately due to its basic protein properties, contrasting with the predominantly acidic nature of other *NLPs* (Figure 4).

DISCUSSION

Plant TFs are an important part of gene regulatory mechanisms where TFs regulate a plethora of genes responsible for a diverse range of morphological, biochemical, and physiological pathways (Liu *et al.,* 2024). A reason why TFs are frequently applicable in reprogramming gene regulatory mechanisms for crop improvement, including biotic and abiotic stress tolerance (Shah *et al.,* 2016; Shinwari *et al.,* 2020). *NLPs* constitute an essential group of plant-specific TFs (Liu *et al.,* 2018). These TFs are central to the regulation of N uptake, assimilation, transport, and overall NUE within plants. They exhibit a nuclear-retention mechanism in response to increased N levels, which allows for the accumulation of *NLP* proteins within the nucleus (Alfatih *et al.,* 2023). This accumulation subsequently enhances the expression of N-responsive genes, thereby optimizing the plant's ability to use N efficiently.

In alignment with the aforementioned understanding, this research focused on mungbean and sought to explore the existence of *NLP*s in them. The availability of the mungbean whole-genome sequence, published in 2018, provides an opportunity to identify and characterize *NLP* genes within this legume. Utilizing genome-wide approaches, the potential study identified seven *VrNLP* genes. Although such genome-wide studies do not offer conclusive evidence regarding the molecular mechanisms occurring within cells, they are invaluable for the initial identification of gene families and the preliminary analysis of their structural and functional properties. In our study, the identified *VrNLPs'* comparison to *AtNLPs* ensued, a model organism extensively studied for N metabolism. The comparative analysis revealed that *VrNLPs* possess longer gene and protein lengths, as well as higher molecular weights, than *AtNLPs*. These structural variations suggest possible differences in the regulatory functions of these genes between the two species. However, the pI and GRAVY of *VrNLPs* were notably similar to those of *AtNLPs*, indicating potential functional conservation between the two gene families. It is evident from previous reports that gene structure evolution is a result of a loss or gain of introns (Zhang *et al.,* 2014). A similar study, conducted by Jan *et al.* (2023), identified a total of six *NLP* genes in the non-vascular model plant *Physcomitrella patens,* which supported the statement that *NLPs* are plant-specific TFs present in a wide range of plants, including both vascular and non-vascular.

Phylogenetic analysis revealed evolutionary relationships between *AtNLPs* and *VrNLPs* through their distribution across five distinct subclades. The formation of a separate *VrNLP* subclade, closely clustered with *NLPs* from *Arabidopsis thaliana* and *Zea mays*, offers important insights into the evolutionary divergence among vascular plants. This phylogenetic pattern likely reflects adaptive specialization of *NLPs* to N metabolic pathways in different plant lineages. The conserved evolutionary relationship between *VrNLPs* and other vascular plant *NLPs* further demonstrates both ancestral origins and functional preservation within this TF family. Supporting evidence comes from the identification of shared protein motifs between *VrNLPs* and *AtNLPs*, reinforcing their conserved roles in fundamental processes like plant growth and development. Study findings align with recent evolutionary studies of amino acid permeases (AAPs) across 17 plant species, which confirmed shared ancestry between bryophytes and vascular plants along with lineage-specific gene duplications (Zhang *et al.,* 2020).

Presented results confirm the presence of *NLPs* in mung beans, which could be beneficial in genetic engineering among both inter- and intra-species. Meanwhile, as in silico analyses provide valuable functional predictions, experimental validation through molecular techniques remains essential—a conclusion supported by previous *NLP* studies in *Physcomitrella patens* demonstrating N-responsive regulation of NUE (Jan *et al.,* 2023). Collectively, these findings significantly advance our understanding of *NLPs'* existence and conservation in plants. They could be potentially helpful for improving crop productivity and overall sustainable agriculture.

CONCLUSIONS

The study verified the presence of *VrNLPs*, suggesting that *VrNLPs* are functionally similar to *AtNLPs* as predicted from their structural attributes studied through in silico tools. In addition to the structural and functional resemblances with *AtNLPs*, *VrNLPs* share evolutionary relationships with both the vascular and non-vascular plants. This approach successfully provided initial insights into the *VrNLP* gene family. Future research should focus on mutant studies for in-depth functional characterization.

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