

Article

Genome-Wide Analysis of *NLP* Genes in Peanut Reveals Significant Roles of *AhNINa* and *AhNINb* in Root Nodule Development

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Abstract

Nitrogen is an indispensable nutrient for plant growth and crop production, but it is not directly accessible to plants without the help of nitrogen-fixing bacteria. Legume plants can form root nodules in symbiosis with rhizobia. NODULE INCEPTION (NIN), a founding member of the NIN-like protein (NLP) family, is essential for nodulation in legume species. However, the knowledge of functional characteristics of the NLP family members in peanuts is limited. In this study, a genome-wide analysis of the *NLP* genes was carried out. A total of 16 *NLP* genes were identified in the peanut genome, including 2 *AhNIN* and 14 *AhNLP*, which were unevenly distributed on nine chromosomes of the peanut genome. Furthermore, transcriptomic profiles and expression pattern analysis showed that both *AhNINa* and *AhNINb* genes were specifically expressed in root nodules. Subcellular localization and transcriptional activity analysis revealed that both *AhNINa* and *AhNINb* encode transcriptional activators. In addition, the roots that down-regulated the expression of *AhNINa* and *AhNINb* genes failed to form nodules. These findings provide significant insights into the molecular functions of *AhNINa* and *AhNINb* genes in regulating peanut nodule development.

Keywords: peanut; *NLP* gene family; *AhNINa*; *AhNINb*; nodule development



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1. Introduction

Nitrogen (N) is an essential nutrient element and plays an important role in the growth and development of plants [1]. However, in the process of agricultural production, only 30% of soil-available nitrogen is absorbed by plant roots in the form of nitrate (NO_3^-) and ammonium (NH_4^+) ions and other organic molecules [2]. The remaining 70% of nitrogen enters the soil through leaching or enters the atmosphere in gaseous form, which will also cause environmental pollution [3]. It was reported that genes and transcription factors that are involved in nitrogen signaling pathways play crucial roles in nitrate uptake and

transport [4]. NIN-like proteins (NLPs) are considered to be a plant-specific transcription factor family and are involved in nitrate signaling response and symbiotic nodulation [5,6].

The NODULE INCEPTION (NIN) protein was the first identified NLP family member in *Lotus japonicus*, which was found to control nitrogen-mediated symbiotic nodule formation [7]. Mutants with loss of *NIN* orthologs function were unable to form nodules in *Glycine max*, *Medicago truncatula*, and *Lotus japonicus* [8–10]. *NLP* genes were named after their homology to *NIN* and were considered to be key regulators of nitrate signaling in land plants [5,11,12]. NLPs are DNA-binding proteins with an N-terminal RWP-RK motif, which binds to the nitrate-responsive *cis*-element (NRE) region of nitrate-responsive genes, and a C-terminal PB1 domain, which is responsible for protein–protein interactions [13]. Previous studies have shown that the NLP transcription factors widely exist in legume and nonlegume plants, such as *Medicago sativa*, *Brassica napus*, and *Oryza sativa* [14–16]. In *Arabidopsis thaliana*, nine NLP members, AtNLP1 to AtNLP9, have been identified, which can all bind the NRE and activate the expression of nitrate-responsive genes [12,17]. Loss of *AtNLP7* function results in plants displaying an N-starvation phenotype due to impaired expression of nitrogen pathway-related genes, such as *ARABIDOPSIS NITRATE REGULATED 1* (*ANR1*), *NITRATE TRANSPORTER 2* (*NRT2*), and *LATERAL ORGAN BOUNDARY DOMAIN 37* (*LBD37*) [18,19]. Moreover, *AtNLP7* overexpression improves plant nitrogen use efficiency and photosynthetic efficiency, thereby promoting plant growth and development [20]. In addition, *AtNLP7* can interact with *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1-20* (*AtTCP20*) to regulate nitrate signaling [21]. Another *NLP* gene, *AtNLP6*, has the highest sequence similarity with *AtNLP7*, but disruption of *AtNLP6* alone does not cause obvious phenotype defects; however, mutation of the *AtNLP6* gene in the *nlp7-1* mutant background exacerbates changes in gene expression and growth defects [21], suggesting that *AtNLP6* and *AtNLP7* play a redundant role while *AtNLP8* regulates nitrate-promoted seed germination by directly binding to the promoter of an abscisic acid catabolic enzyme encoding gene *CYTOCHROME P450 MONOOXYGENASE 707A2* (*AtCYP707A2*) and reducing abscisic acid levels in a nitrate-dependent manner [22]. Furthermore, *AtNLP2* regulates rosette leaf growth and root development in response to changes in nitrate availability [23]. Therefore, NLP transcription factors play important roles in plant nitrogen metabolism, leaf and root development, and nodule formation.

Legumes are able to establish root nodule symbiosis with nitrogen-fixing soil bacteria, which are collectively called rhizobia. The root nodule symbiosis establishment involves the invasion of rhizobia in root epidermis and the formation of nodules through the root cortical cells. The most common mode of rhizobia invasion in legume, such as *Vicia* sp., *Trifolium* sp., and *Pisum* sp., is root hair infection [24]. Peanut (*Arachis hypogea* L.) is an economically important allotetraploid legume crop, serving as a source of edible vegetable oil and protein. Worldwide, peanuts are grown in 27.66 million hectares in 84 countries with an annual production of 43 million tons of pods (i.e., nuts-in-shell), with the 1590 kg ha^{−1} productivity. The largest producers in the world are China, India, and the United States and exports are approximately 1.25 million metric tons. Peanut can form root nodules with cognate *Bradyrhizobium* spp. Rhizobia enter peanut roots using developmental cracks called “crack-entry” and generate aescynomenoid-type determinate nodules containing a uniform infection zone [25–27]. In peanut, rhizobia can directly penetrate into the root cortex through cracks at lateral root bases or wounding sites [28]. Previous studies have shown that the receptor-encoding gene *NOD FACTOR RECEPTOR 5* (*AhNFR5*) and several symbiotic signaling genes, such as *Ca*²⁺/*CALMODULIN DEPENDENT KINASE* (*AhCCaMK*), *HISTIDINE KINASE 1* (*AhHK1*), and *NODULATION SIGNALING PATHWAY 2* (*AhNSP2*),

play key roles in peanut nodulation [29–32]. However, the molecular function of the *NLP* gene family in peanut nodulation is largely unknown.

In this study, a genome-wide analysis of the *NLP* gene family in peanuts was carried out. A total of 16 *NLP* genes, including 2 *AhNIN* and 14 *AhNLP*, were identified in the peanut genome, and their chromosome locations, phylogenetic relationship, protein motifs, gene structure, and transcriptome profile levels in different organs were analyzed. Furthermore, we found that *AhNINa* and *AhNINb* are specifically expressed in root nodules. Simultaneous down-regulation of both *AhNINa* and *AhNINb* by RNA interference (RNAi) led to the failure of nodule formation. Therefore, this study provides detailed insights into the classification of *NLPs* and an in-depth understanding of the molecular function of *AhNINa* and *AhNINb* genes in nodule development in peanuts.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The cultivated peanut variety “Shitouqi” was used as the experimental material in this study. The seeds of “Shitouqi” were collected by Shandong Peanut Research Institute (Qingdao, China). The seeds were planted into seedling pots (5 × 5 × 8 cm length, width, height) with sterilized vermiculite and grown in a growth chamber (day: 16 h, 25 °C; night: 8 h, 23 °C; relative humidity: 70%).

2.2. Root Nodule Induction

For nodule induction, the peanut seeds were planted into sterilized vermiculite. After five days, the germinated seedlings were treated with 2 mL of commercial rhizobia strain suspension (*Bradyrhizobium* sp. HHPB1; Colony-Forming Units (CFUs) ≥ 3 million/mL; Anhui New Simon Biotech Co., Ltd., Hefei, China) and grown in a growth chamber.

2.3. Hairy Root Transformation

For peanut hairy root transformation, the *A. rhizogenes* strain K599 containing the plasmids was transformed into peanut hypocotyl as described previously [33]. Briefly, healthy peanut seedlings at 7–10 days post-germination, with fully expanded cotyledon, were selected for transformation. The primary root was removed using sterile scissors, leaving a 0.5–0.8 cm segment of the hypocotyl intact. The wound site was thoroughly coated with *Agrobacterium rhizogenes* strain K599 harboring the corresponding vector, and the seedlings were directly inserted into moist sterilized vermiculite and grown in a growth chamber. After approximately four weeks, the transgenic hairy roots could be visually identified. Throughout this period, vermiculite moisture was consistently maintained to ensure successful root induction.

2.4. Identification of the *AhNIN* and *AhNLP* Genes in Peanut

To identify the *AhNIN* and *AhNLP* genes in the peanut genome, 1 MtNIN, 5 MtNLP, and 9 AtNLP protein sequences were used to search for their orthologs in the *A. hypogaea* genome database (<http://peanutgr.fafu.edu.cn>, accessed on 15 October 2023), with an E-value ≤ 1 × 10^{−5}. The *Arabidopsis* AtNLP genes were obtained from the TAIR website (www.arabidopsis.org/, accessed on 15 October 2023) and the *M. truncatula* MtNIN and MtNLP genes were obtained from the MtrunA17 database (<https://medicago.toulouse.inra.fr>, accessed on 15 October 2023). In total, 2 *AhNIN* and 14 *AhNLP* genes were identified in *A. hypogaea* genome using blast with an E-value ≤ 1 × 10^{−5}.

The physicochemical properties of *AhNLP* and *AhNIN* proteins, including molecular weight (Mw) and isoelectric point (pI) were predicted using ExPASy ProtParam (<http://web.expasy.org/protparam/>, accessed on 3 June 2024). The subcellular localizations of

AhNLP and AhNIN proteins were predicted using Cell-PLoc 2.0 (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>, accessed on 27 May 2025).

2.5. Multiple Sequence Alignment and Phylogenetic Analysis of the NIN and NLP Genes

Multiple protein sequence alignment was performed using Jalview 2.10.5 software (www.jalview.org/, accessed on 25 March 2023). The phylogenetic tree of NIN and NLP proteins from *A. hypogaea*, *A. thaliana*, and *M. truncatula* was constructed by MEGA 7.0 using the neighbor-joining method with the following parameters: Poisson correction, pair-wise deletion, and bootstrap values in percentages with 1000 replicates.

2.6. Gene Structure, Chromosome Location, and Conserved Motif Detection

To analyze the gene structures of the *AhNIN* and *AhNLP* genes, the genomic sequences and CDS sequences were obtained from the peanut genome database. Then, the *AhNIN* and *AhNLP* genomic sequences and CDS sequences were aligned using the gene structure display server 2.0 (GSDS) website (<http://gsds.cbi.pku.edu.cn/>, accessed on 12 May 2025) to generate the diagrams of exon–intron structures.

For chromosome location analysis, the *AhNIN* and *AhNLP* gene information, including gene length and gene location, on the chromosome was obtained from the peanut genome database. The MapGene website (http://mg2c.iask.in/mg2c_v2.1/, accessed on 12 May 2025) was used to visualize the chromosomal distribution of these genes in the peanut genome.

Conserved motifs and domains in AhNIN and AhNLP proteins were analyzed with the online Multiple Em for Motif Elicitation (MEME) program (<http://meme-suite.org/>, accessed on 15 May 2025). The optimized MEME parameters were as follows: repetition number, any; maximum motif width, 200; minimum motif width, 6; and maximum motif number, 20. Multiple protein sequences alignment was carried out with Jalview software (<https://jalview.software.informer.com/>, accessed on 15 May 2025).

2.7. RNA Extraction and Reverse Transcription Quantitative PCR (RT-qPCR)

To detect the relative expression levels of *AhNINa* and *AhNINb* genes in different developmental stage nodules, nodules of 7, 14, and 21 days post-inoculation (dpi) with rhizobia were collected and the total RNA was extracted. Roots without inoculation with rhizobia were used as the control (0 dpi). Briefly, 20 seeds of cultivated peanut variety “Shitouqi” were planted into sterilized vermiculite. After five days of sowing, the roots of five peanuts were collected to form a pool as the control (0 dpi). Then, 2 mL of commercial rhizobia strain suspension was applied to each of the remaining peanut seedlings. Seven days post-inoculation (dpi) with rhizobia, the nodules from five peanut roots were collected to form a pool as the seven dpi experimental group. Nodules at 14 dpi and 21 dpi were also obtained by a similar method to the 7 dpi experimental group. To analyze the relative expression levels of *AhNINs* genes in *AhNINs-RNAi* transgenic hairy roots, each independent hairy root was collected after four weeks inoculation with rhizobia. The samples of roots and different developmental stage nodules were taken off and quickly stored in liquid nitrogen. Then, the total RNA of different samples was extracted using the Trizol-RT Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer’s instructions. Two micrograms of total RNA from each organ/tissue were reverse-transcribed into cDNA using the HiScript IV All-in-One Ultra RT SuperMix Kit (Vazyme, Nanjing, China). The RT-qPCR analysis was performed on Bio-Rad CFX Connect™ using 2X M5 HiPer Realtime PCR mix (Mei5bio, Beijing, China). The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The *AhG6PD* gene was selected as an internal control for normalization [34].

2.8. Vector Construction, Subcellular Localization, and Promoter-GUS Analysis

For subcellular localization analysis, the CDS of *AhNINa* and *AhNINb* genes were PCR-amplified using gene-specific primer pairs *AhNINa*-CDS-F and *AhNINa*-CDS-R and *AhNINb*-CDS-F and *AhNINb*-CDS-R, respectively (Supplementary Table S1). Then, the PCR products were purified and cloned into the *pENTR/D TOPO* vector to generate the *AhNINa:pENTR* and *AhNINb:pENTR*, respectively. Finally, the *pro35S:AhNINa-GFP* and *pro35S:AhNINb-GFP* constructs were generated by gateway recombination reaction between *pEarleyGate 103* and *AhNINa:pENTR*, and *pEarleyGate 103* and *AhNINb:pENTR*, respectively [35]. For tobacco transformation, three-weeks-old *Nicotiana benthamiana* leaves were injected with *Agrobacterium* GV3101 (pSoup-p19) strain suspension with OD₆₀₀ = 0.8 containing the *pro35S:AhNINa-GFP* and *pro35S:AhNINb-GFP* plasmids, respectively. After incubation in the dark for 24 h and then in the light for 36 h, the leaves were dissected for observation. For fluorescent imaging, a Leica LSM 880 laser scanning confocal microscope was used. The 488 nm line of an argon laser was chosen for the green fluorescent protein signal.

For gene promoter expression pattern analysis, the 1483 bp and 1474 bp promoter sequences of *AhNINa* and *AhNINb* genes were PCR-amplified using gene-specific primer pairs *AhNINa*-Pro-F and *AhNINa*-Pro-R and *AhNINb*-Pro-F and *AhNINb*-Pro-R, respectively (Supplementary Table S1). Then, the promoter sequences of *AhNINa* and *AhNINb* were subcloned into the *pENTR/D TOPO* vector to generate the *proAhNINa:pENTR* and *proAhNINb:pENTR*, respectively. Finally, the *proAhNINa:GUS* and *proAhNINb:GUS* constructs were generated by gateway recombination reaction between the gateway vectors *pBGWFS7* and *proAhNINa:pENTR*, and *pBGWFS7* and *proAhNINb:pENTR*, respectively [36].

2.9. RNAi Analysis

For *AhNINa* and *AhNINb* genes RNAi analysis, a common 308 bp CDS sequence of both *AhNINa* and *AhNINb* was cloned and subcloned into the *pENTR/D TOPO* vector to generate the *AhNINab:pENTR*. Then, the *AhNINab:pENTR* vector underwent a gateway recombination reaction with gateway vector *pH7GWIWG2D (II)* to generate the RNAi construct [36]. For root phenotype analysis, the RNAi-transformed roots were selected by eGFP fluorescence observed under Leica stereo-fluorescence microscope M205FA equipped with a Leica 34 DFC310 FX digital camera (Leica Microsystems, Wetzlar, Germany).

2.10. Transcriptional Activation Activity Analysis and Yeast Cell Transformation

For transcriptional activation activity analysis, the CDS of *AhNINa* and *AhNINb* genes were cloned using primer pairs *AhNINa*-CDS-F and *AhNINa*-CDS-R and *AhNINb*-CDS-F and *AhNINb*-CDS-R, respectively (Supplementary Table S1). Then, the PCR products were cloned into the *pENTR/D TOPO* vector to generate the *AhNINa:pENTR* and *AhNINb:pENTR* vectors. Finally, the *pGBKT7* vector underwent a gateway recombination reaction with the *AhNINa:pENTR* and *AhNINb:pENTR*, respectively, to generate the destination vectors *AhNINa:pGBKT7* and *AhNINb:pGBKT7*. For yeast cell transformation, vector combinations *pGADT7* and *AhNINa:pGBKT7*, and *pGADT7* and *AhNINb:pGBKT7* were transformed into yeast (*Saccharomyces cerevisiae*) strains AH109. Then, the yeast cells containing the corresponding vectors were selected on DDO (SD/-Leu-Trp) medium and examined on TDO/X (SD/-His-Leu-Trp with X-a-Gal) according to the manufacturer's protocol (Matchmaker, Clontech Laboratories, Mountain View, CA, USA).

2.11. Statistical Analysis

All the data are expressed as the means $\pm$ standard deviations (SDs) using Excel 2021. GraphPad Prism 5.0 was used to map the final data, and differences among gene expression was examined by Student's *t*-test (***) $p < 0.001$.

3. Results

3.1. Genome-Wide Identification of NLP Genes in Peanut

To identify the NLP genes in peanut, a BLASTP search using the *A. thaliana* and *M. truncatula* NLP genes against the *A. hypogaea* database (<http://peanutgr.fafu.edu.cn>, accessed on 15 October 2023) was executed. A total of 16 NLP genes, including 2 *AhNIN* and 14 *AhNLP*, were obtained in the *A. hypogaea* genome (Table 1, Supplementary Data S1). These *AhNLP* genes were named based on their closest *M. truncatula* orthologs. The gene characteristics, as well as the gene names, accession numbers, protein lengths, molecular weight (MW), isoelectric point (pI), and predicted subcellular locations are summarized in Table 1. The protein sequence lengths of the 16 identified *AhNIN* and *AhNLP* genes ranged from 577 to 984, their MWs ranged from 64.09 to 109.25 kDa, and their pIs ranged from 4.94 to 8.84. The predicted subcellular localization results showed that most of the *AhNIN* and *AhNLP* genes were localized in the nucleus or chloroplasts, and the others were localized both in the chloroplasts and nucleus.

Table 1. Characterization of *AhNIN* and *AhNLP* genes in peanut.

Gene Name	Accession Number	Length (aa)	MW (kDa)	pI	Predicated Localization
<i>AhNINa</i>	AH13G60030.1	865	95.61	6.33	Nucleus
<i>AhNINb</i>	AH17G00510.1	862	95.39	6.37	Nucleus
<i>AhNLP1a</i>	AH08G02220.1	964	107.48	5.77	Nucleus
<i>AhNLP1b</i>	AH17G22700.1	912	101.89	5.99	Nucleus
<i>AhNLP2a</i>	AH03G04320.1	577	64.09	7.63	Chloroplast
<i>AhNLP2b</i>	AH13G06340.1	612	67.84	7.65	Chloroplast
<i>AhNLP2c</i>	AH03G04310.1	863	95.92	5.03	Chloroplast/Nucleus
<i>AhNLP2d</i>	AH03G04850.1	790	87.87	4.86	Chloroplast
<i>AhNLP2e</i>	AH10G03170.1	782	86.77	5.90	Chloroplast
<i>AhNLP2f</i>	AH13G04800.1	899	99.32	5.23	Chloroplast/Nucleus
<i>AhNLP2g</i>	AH13G06330.1	899	99.50	5.07	Chloroplast/Nucleus
<i>AhNLP3a</i>	AH08G11530.1	870	96.79	6.78	Nucleus
<i>AhNLP3b</i>	AH18G02990.1	870	96.85	6.68	Nucleus
<i>AhNLP4a</i>	AH09G24840.1	967	107.52	5.70	Nucleus
<i>AhNLP4b</i>	AH19G43090.1	969	107.84	5.75	Nucleus
<i>AhNLP5</i>	AH15G36260.1	984	109.25	5.48	Nucleus

3.2. Phylogenetic Analysis and Chromosomal Locations of the *AhNIN* and *AhNLP* Genes

To explore the evolutionary relationships within the *AhNIN* and *AhNLP* genes, a phylogenetic tree was constructed using the 31 proteins, including 2 *AhNIN*, 14 *AhNLP*, 1 *MtNIN*, 5 *MtNLP*, and 9 *AtNLP*, from *A. hypogaea*, *M. truncatula*, and *A. thaliana*. Based on the phylogenetic analysis, the 31 proteins were divided into three different clades: clade 1 (the *AhNIN* and *AhNLP1* clade), clade 2 (the *AhNLP4* and *AhNLP5* clade), and clade 3 (the *AhNLP2* and *AhNLP3* clade) (Figure 1). The *AhNIN* and *AhNLP1* clade and the

AhNLP4 and AhNLP5 clade contain four and three proteins, respectively. However, the AhNLP2 and AhNLP3 clade was the largest group with nine members.

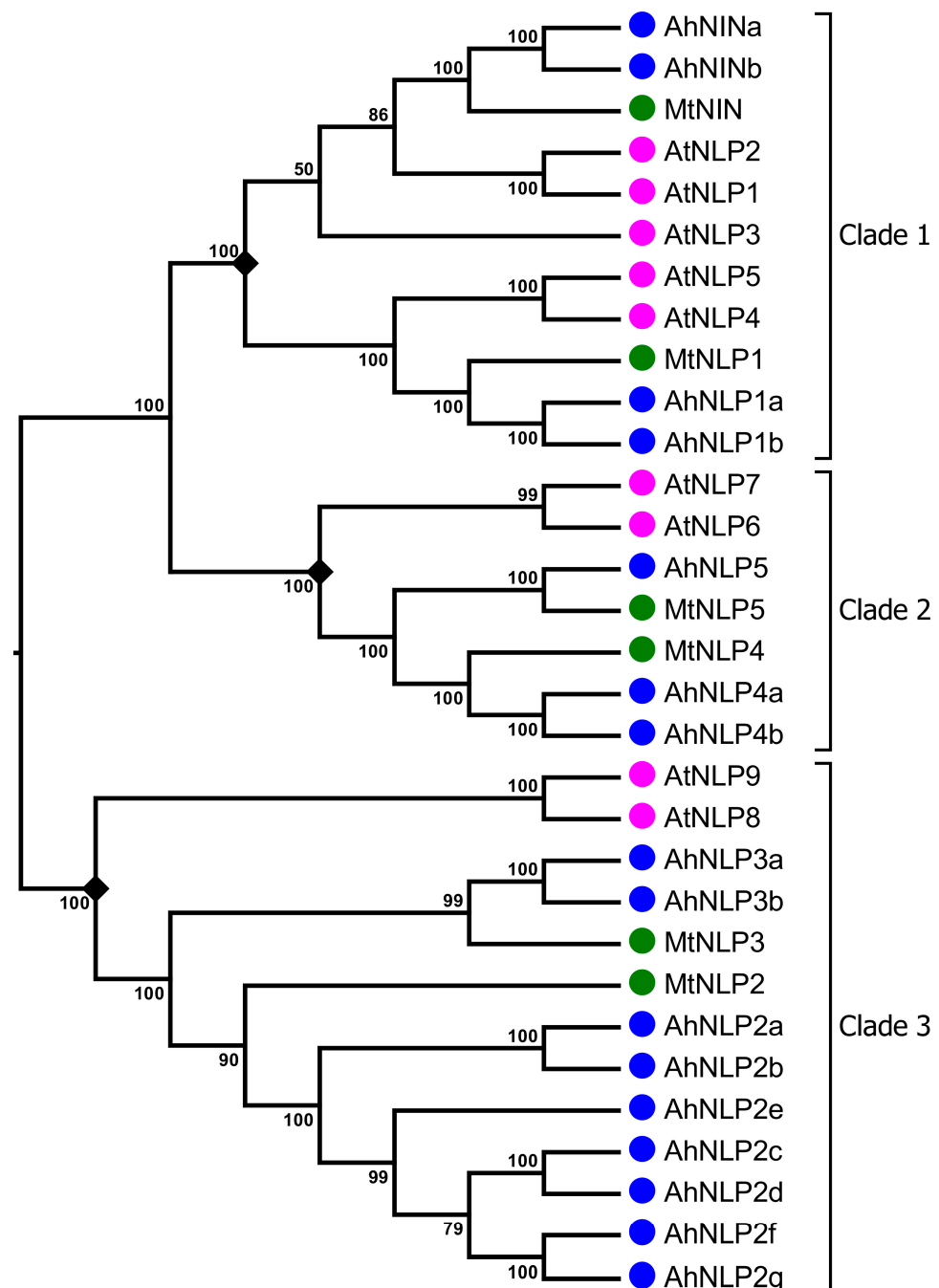


Figure 1. Phylogenetic analysis of *NIN* and *NLP* genes in *A. hypogaea* (Ah), *A. thaliana* (At), and *M. truncatula* (Mt). The neighbor-joining (NJ) phylogenetic tree was constructed using 31 protein sequences from *A. hypogaea* (16), *A. thaliana* (9), and *M. truncatula* (6) in MEGA 7.0 with 1000 bootstrap replicates. The *NIN* and *NLP* genes in *A. hypogaea*, *A. thaliana*, and *M. truncatula* are classified with blue, pink, and green dots, respectively. The black rhombuses in the phylogenetic tree indicate the different clades.

The chromosomal localization information related to *AhNIN* and *AhNLP* genes was retrieved from the *A. hypogaea* genome database. Chromosome distribution analysis showed that the 2 *AhNIN* and 14 *AhNLP* genes were unevenly distributed on nine chromosomes of the *A. hypogaea* genome (Figure S1). The number of genes on chromosome 13 was the largest with four genes. Furthermore, the chromosomes 8 and 17 each have two genes,

respectively. The other chromosomes, such as chromosomes 9, 10, 15, 18, and 19, contain only one member, respectively.

3.3. Conserved Motifs and Domains in AhNIN and AhNLP Proteins

To obtain a better understanding of the protein characteristics of the AhNIN and AhNLP, a MEME search was executed to analyze the conserved motifs and domains in AhNIN and AhNLP proteins. Based on the length of the motifs and domains, the AhNIN and AhNLP proteins all contained ten different motifs/domains (Figures 2 and S2). The motifs 1, 2, 3, and 4 were identified as the most conserved motif, which was present in every AhNIN and AhNLP protein. It was reported that RWP-RK domain and PB1 domain are distinctive domains of *NLP* genes [37,38]. Sequence alignment revealed that all the AhNIN and AhNLP proteins contain the conserved RWP-RK domain (motif 1) and the PB1 domain (motif 3) (Figure S3). However, motif 10 was only identified in the N-terminal of AhNLP2d, AhNLP2e, and AhNLP2f (Figure 2), indicating that this motif is related to the functions of these AhNLP proteins.

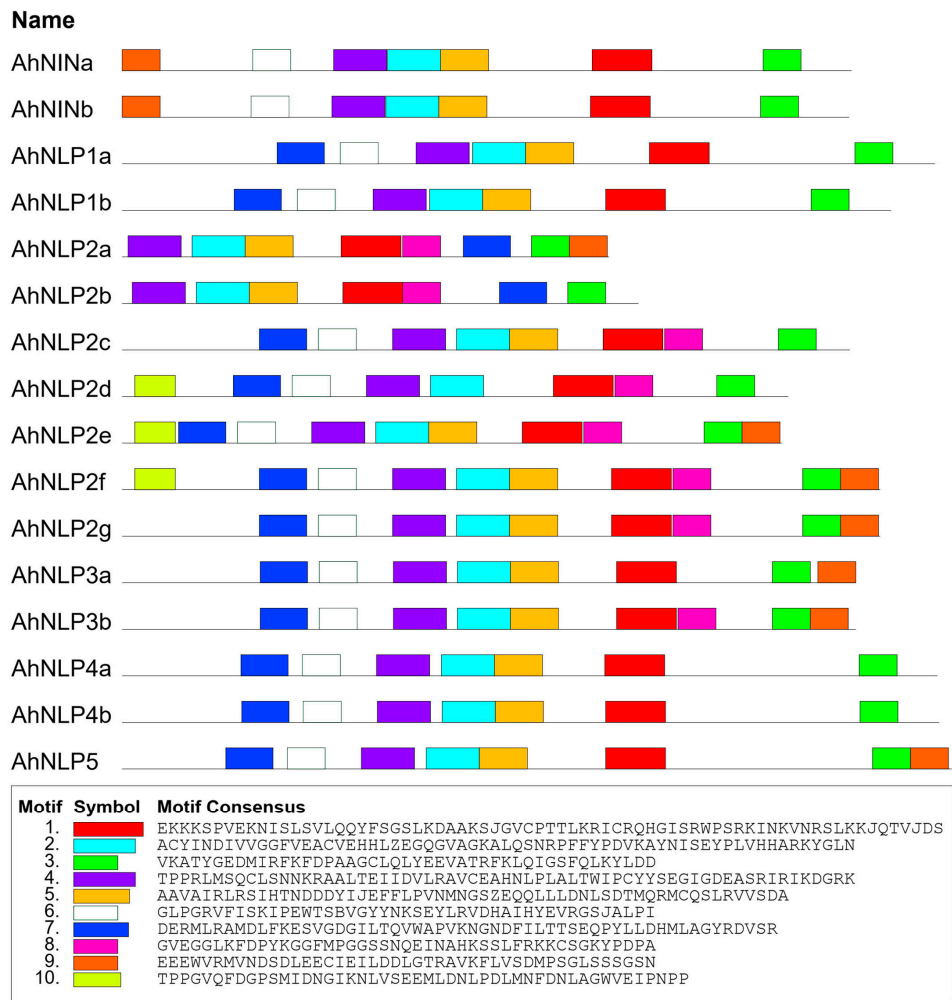


Figure 2. Motif composition of the AhNIN and AhNLP proteins. The conserved motifs are predicted by online MEME website (<https://meme-suite.org/meme/>). The different motifs are represented by boxes with different colors. Motif 1 and motif 3 represent the RWP-RK domain and the PB1 domain.

3.4. Gene Structure of the AhNIN and AhNLP Genes

To further understand the structural diversity of the peanut *AhNIN* and *AhNLP* genes, the gene exon–intron organization analysis was carried out using the online Gene Structure Display Server tool (<http://gsds.cbi.pku.edu.cn/>, accessed on 12 May 2025) (Figure 3). The

exon–intron structures of the 2 *AhNIN* and 14 *AhNLP* genes were generated by the alignment of their gene coding sequences with their corresponding genomic sequences. Gene structure analysis showed that the *AhNLP2d* gene has no 5' upstream and 3' downstream sequences, and *AhNLP2a* gene has no 5' upstream sequence. Furthermore, the clade 2 *NLP* genes, *AhNLP4a*, *AhNLP4b*, and *AhNLP5*, have similar exon–intron organization. However, the exon numbers in clade 3 (the *AhNLP2* and *AhNLP3* clade) *NLP* genes changed from three to eight, indicating that these *AhNLP2* and *AhNLP3* genes may play differential functions during peanut growth and development.

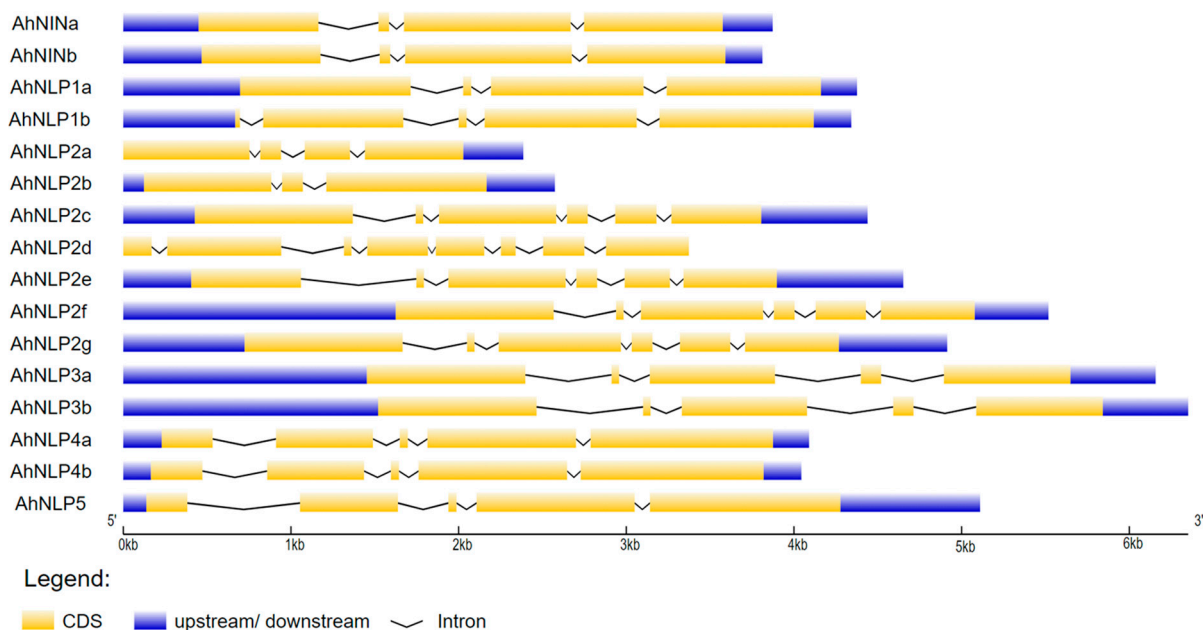


Figure 3. The gene structures of the *AhNIN* and *AhNLPs* genes. The 5' UTRs and 3' UTRs are represented by blue boxes; the exons and introns are represented by yellow boxes and black folded lines. The scale was referred to the lengths of the genes. The exon–intron structures were identified by online Gene Structure Display Server tool (<http://gsds.cbi.pku.edu.cn/>, accessed on 12 May 2025).

3.5. Expression Profiles of the *AhNIN* and *AhNLP* Genes

To examine the expression patterns of *AhNIN* and *AhNLP* genes, the expression profiles of the *AhNIN* and *AhNLP* genes in different organs and tissues of cultivated peanut variety “Shitouqi” were analyzed using publicly available transcriptome sequencing data (<http://peanutgr.fafu.edu.cn>, accessed on 1 April 2024) (Figure 4). The heat map showed that the *AhNLP2e*, *AhNLP2g*, *AhNLP4a*, and *AhNLP4b* genes were highly expressed in all tissues or organs, suggesting that they may play important roles in peanut growth and development while the other *AhNLP* genes were relatively lower-expressed in all tissues or organs. However, *AhNINa* and *AhNINb* were specifically highly expressed in root nodules, indicating that *AhNINa* and *AhNINb* may play crucial roles in nodule formation and development in peanut.

3.6. Nodule-Specific Expression Patterns of *AhNINa* and *AhNINb*

To gain better insights into the molecular functions of *AhNINa* and *AhNINb* genes in peanut nodule development, the expression patterns of both *AhNINa* and *AhNINb* were analyzed in detail. First, the relative expression levels of *AhNINa* and *AhNINb* in nodules at different developmental stages were measured. Nodules were harvested at 7, 14, and 21 day post-inoculation (dpi) with rhizobia strain, and roots without inoculation with rhizobia were used as the control (0 dpi). Due to the high sequence identity between *AhNINa* and *AhNINb* genes, we failed to design specific reverse transcription quantitative PCR

(RT-qPCR) primers for individual *AhNINa* and *AhNINb* genes, and quantified consolidated expression of both *AhNINs*. RT-qPCR data showed that *AhNINs* were highly expressed at different developmental stages of nodules, compared with those in the root of 0 dpi (Figure 5A). Second, to analyze the expression patterns of *AhNINa* and *AhNINb* in nodules in more detail, the *proAhNINa:GUS* and *proAhNINb:GUS* hairy root transgenic plants were obtained. GUS staining showed that *AhNINa* and *AhNINb* showed a similar expression pattern and were specifically expressed in nodules (Figure 5B–E). These results demonstrate that both *AhNINa* and *AhNINb* are specifically expressed in nodules and may be involved in the development of peanut nodules.

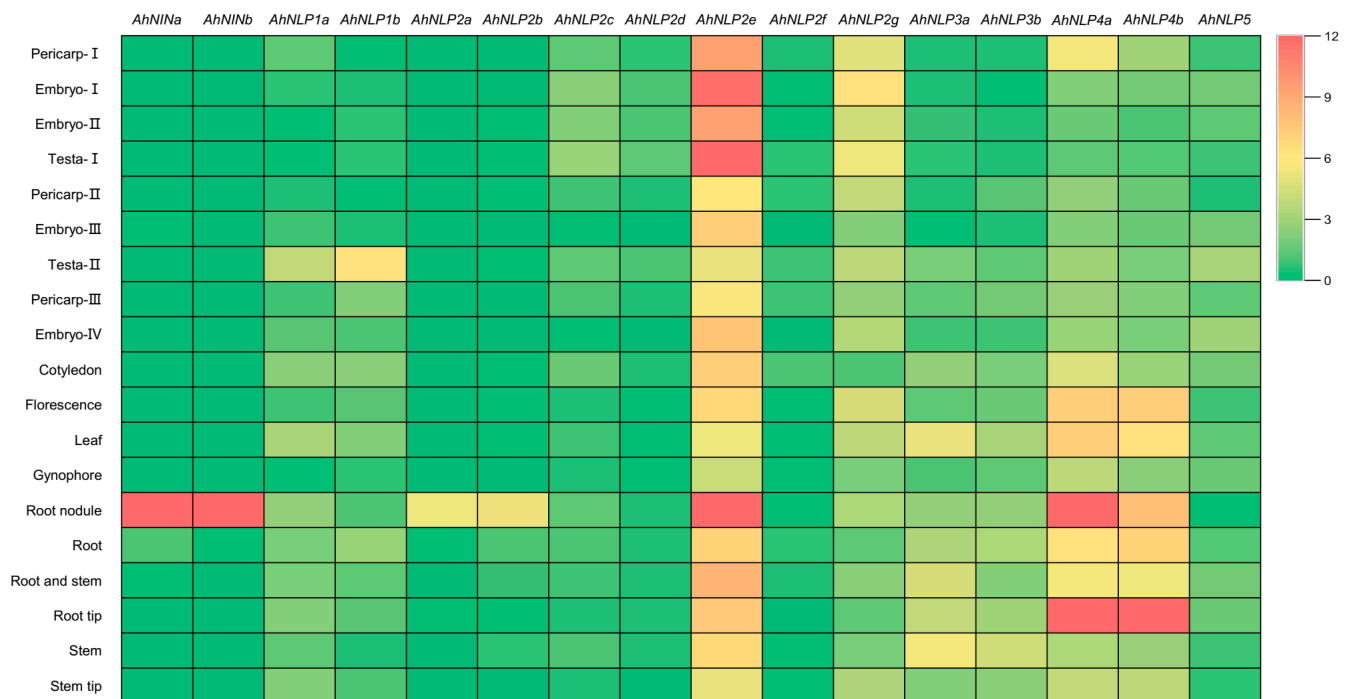


Figure 4. Expression profile of *AhNIN* and *AhNLP* genes in different organs and tissues of peanut. The expression heat map was constructed based on FPKM values in the *A. hypogaea* transcriptome dataset (<http://peanutgr.fafu.edu.cn>, accessed on 1 April 2024). The red represents high expression levels and the green represents low expression levels.

3.7. Both *AhNINa* and *AhNINb* Act as Transcriptional Activators

To further evaluate the possible function of *AhNINa* and *AhNINb* genes in peanut nodule development, the subcellular localization analysis of the *AhNINa* and *AhNINb* genes was performed. *AhNINa* and *AhNINb* were, respectively, fused with GFP and transformed into tobacco epidermal cells. Green fluorescence signal observation showed that compared with free GFP which is located in the cell nucleus and cytoplasm (Figure 6A), both *AhNINa* and *AhNINb* were located in the cell nucleus (Figure 6B,C), demonstrating that they are nuclear-localized transcription factors.

To assess the possible transcriptional activity of *AhNINa* and *AhNINb* proteins, yeast transactivation assays were performed. First, the *AhNINa* and *AhNINb* were fused with the GAL4 DNA-binding domain (BD) to generate the *AhNINa:BD* and *AhNINb:BD* constructions, respectively. Then, the GAL4 DNA-activation domain (AD) vector was cotransformed with *AhNINa:BD* or *AhNINb:BD* into yeast cells. Compared to the AD+BD control, the *AhNINa* or *AhNINb* protein alone can activate transcription (Figure 7). These results suggest that *AhNINa* and *AhNINb* may function as transcriptional activators to regulate the nodule development in peanut.

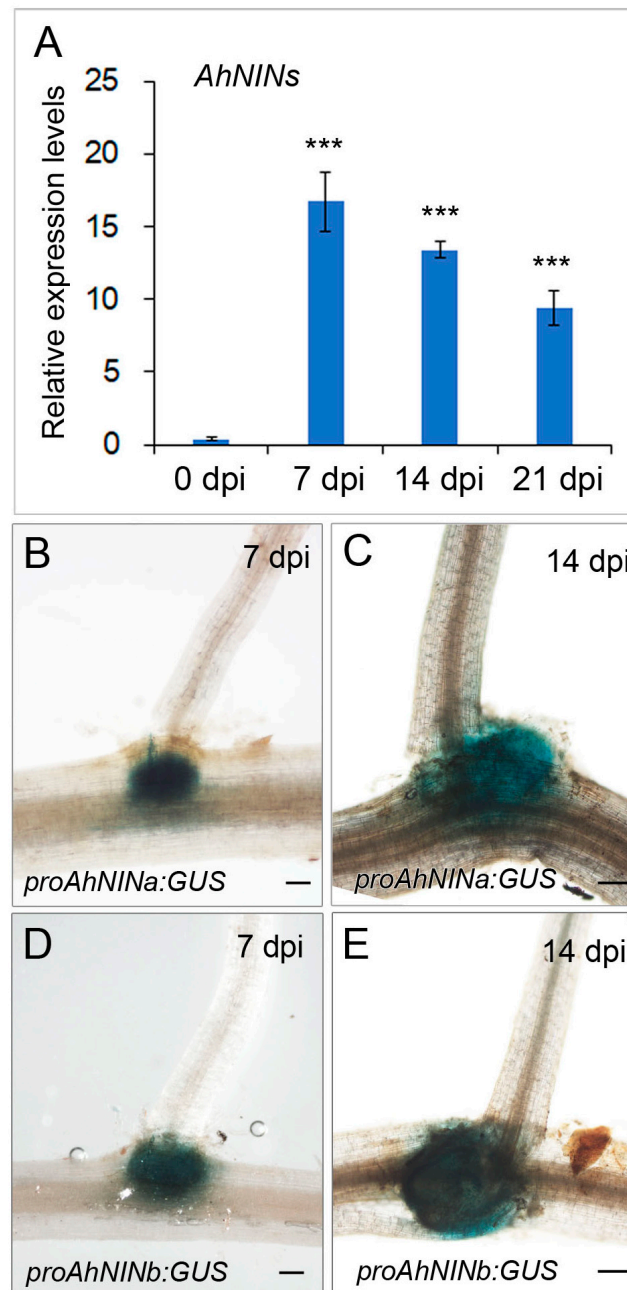


Figure 5. Expression pattern of *AhNINa* and *AhNINb* genes in peanut nodules. **(A)** The relative expression levels of both *AhNINa* and *AhNINb* genes in different developmental stage nodules. Values are the means and SDs of three biological replicates; *** $p < 0.001$. The two-sided Student's *t*-test was used to estimate if the difference is significant. **(B–E)** Promoter-GUS fusion studies of *AhNINa* (**B,C**) and *AhNINb* (**D,E**) expression in hairy root transformed nodules at 7 and 14 dpi. Dpi, days post-inoculation. Scale bars, 500 μ m in (**B–E**).

3.8. *AhNINa* and *AhNINb* Are Required for Nodule Formation in Peanut

To determine whether *AhNINa* and *AhNINb* genes are involved in peanut nodule development, we knocked down the expression of *AhNINa* and *AhNINb* using an RNAi construct that targets the common sequence of both *AhNINa* and *AhNINb* (Figures 8A and S4). RT-qPCR analysis showed that the expression of *AhNINs* was reduced by 92–96% in the RNAi roots compared to the control (EV) (Figure 8B). The root nodule phenotype observation indicated that, compared to the EV control which has nodules (Figure 8C), the *AhNINs*-RNAi transgenic hairy roots with significantly lower gene expression levels had no nodule

formation (Figure 8B,D–F). These results demonstrate that both *AhNINa* and *AhNINb* genes are functional transcription factors and are necessary for nodule formation in peanut.

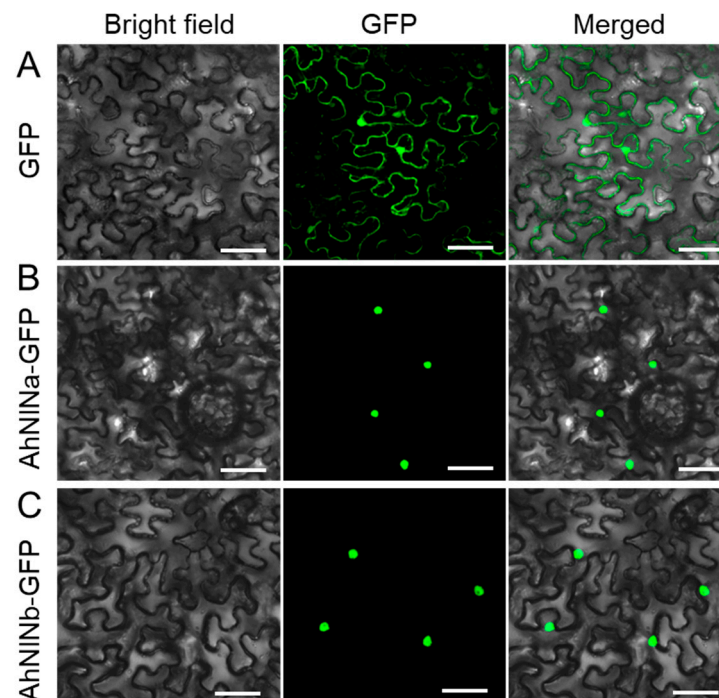


Figure 6. Subcellular protein localization of *AhNINa* and *AhNINb* genes. (A) The subcellular localization of free GFP protein. (B,C) The subcellular localization of AhNINa-GFP (B) and AhNINb-GFP (C) fusion proteins. Both AhNINa-GFP and AhNINb-GFP fusion proteins were localized in the nucleus of tobacco epidermal cells as observed by confocal microscopy. Free GFP was used as a control. Scale bars = 50 μ m.

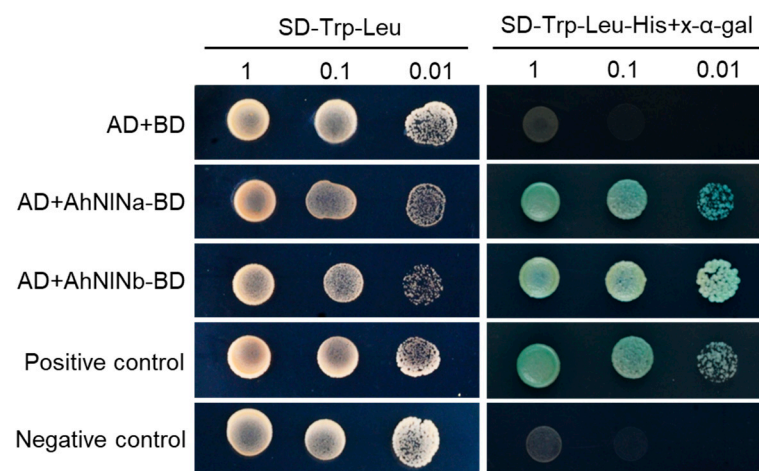


Figure 7. Analysis of the transcriptional activity of *AhNINa* and *AhNINb* in yeast cells. The transcriptional activation activities of *AhNINa* and *AhNINb* were assessed by the growth of yeast cells on DDO (SD/-Leu-Trp) medium and TDO/X (SD/-His-Leu-Trp) medium supplemented with x- α -gal.

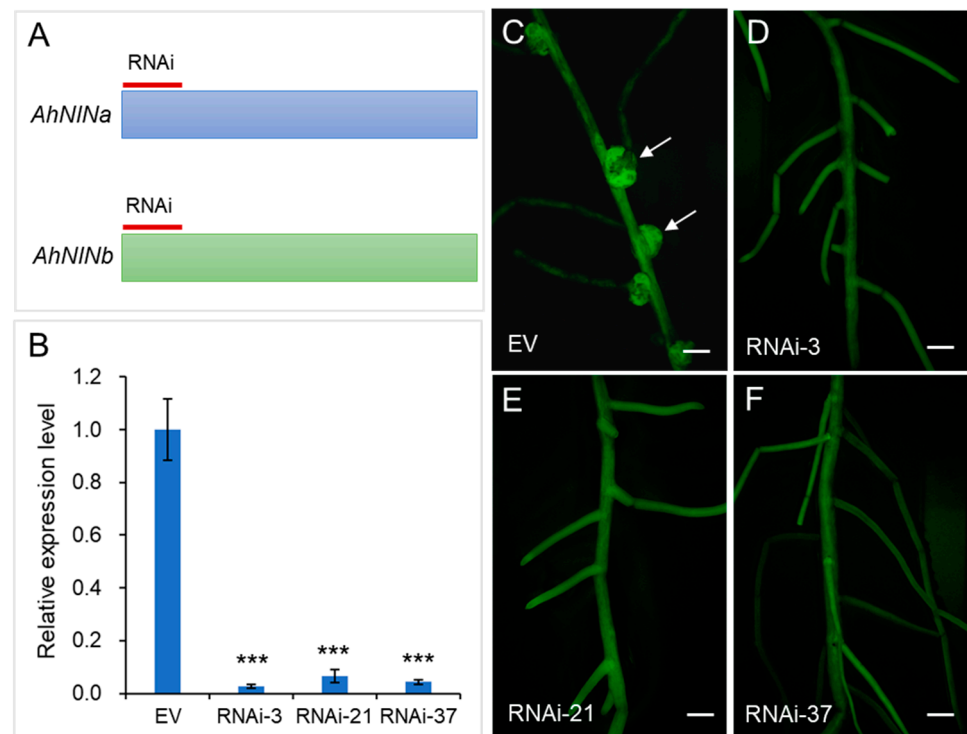


Figure 8. *AhNINa* and *AhNINb* are involved in symbiotic root nodule development in peanuts. (A) Schematic illustration of *AhNINa* and *AhNINb* genes encoding sequence and the red lines indicate the same RNAi sequence in both *AhNINa* and *AhNINb*. (B) RT-qPCR analysis shows the relative expression of the *AhNINs* genes in control (EV) and *AhNINs*-RNAi roots at 28 dpi. Values are the means and SDs of three technical replicates; *** $p < 0.001$. (C–F) Fluorescence phenotype images of EV and *AhNINs*-RNAi roots at 28 dpi. Arrows in (C) mark the root nodules at the base of the lateral roots. Scale bars, 2 mm in (C–F).

4. Discussion

Previous studies have identified 9 *AtNLPs* in Arabidopsis (*A. thaliana*) [12], 6 *OsNLPs* in rice (*Oryza Sativa* L.) [16], 6 *MtNLPs* in Medicago (*M. truncatula*) [39], 9 *ZmNLPs* in maize (*Zea mays* L.) [40] and 18 *TaNLPs* in wheat (*Triticum aestivum* L.) [41]. However, the information and function of the *NLP* genes in peanuts remain largely unknown. In this study, a total of 16 *AhNLPs* were identified in peanut genomes based on the annotation information. Compared with the number of *NLP* genes in Arabidopsis, rice, maize and Medicago, which are all diploid species, the number of the peanut *NLP* gene family members is about twice that of each species, suggesting that the *NLP* gene family of peanut may have undergone significant gene expansion (Table 1; Figure 1). The cultivated peanut (*Arachis hypogaea* L.) is of hybrid origin (two sets of ten chromosomes; mostly $2n = 2 \times = 20$ chromosomes) and has a polyploid genome that contains essentially complete sets of chromosomes from two ancestral species [42,43]. The number of *NLP* genes in peanut was similar to that in hexaploidy wheat, indicating that the increase in the number of *NLP* genes in peanut may be due to polyploidization. In peanut, the Medicago *MtNLP2* gene has seven orthologs, from *AhNLP2a* to *AhNLP2g*, implying that these genes may play a redundant role or gain some special functions in regulating the growth and development of peanut.

Phylogenetic and gene structure analysis showed that the peanut *NLP* family members belong to three different clades (Figure 1) and genes in the same clade display similar gene structures, except clade 3 (Figure 3). It was reported that clade 2 and 3 *NLP* genes originated from mosses and green algae, respectively, while group 1 *NLPs* appeared after

the separation of monocots and dicots [6]. In *Arabidopsis*, the group 3 NLP gene, *AtNLP7*, is regulated by nitrate, and then *AtNLP7* induces the expression of nitrate signaling and assimilation-related genes, such as *AtNRT2.1* and *NITRITE REDUCTASE (AtNIR)* [12,19]. In peanut, *AhNLP4a*, *AhNLP4b*, and *AhNLP5* are the closest orthologs to *AtNLP6* and *AtNLP7*, indicating that they may play a similar function with *AtNLP6* and *AtNLP7*, and participate in the nitrogen-regulated plant growth and development. The RWP-RK and PB1 domains enable NLP to function in multiple aspects of nitrogen metabolism, including nitrogen response, transcriptional regulation, and signal transduction [44,45]. All the *AhNLP* proteins contain the two conserved domains, RWP-RK and PB1 (Figure 2), which are consistent with the structural characteristics of NLP proteins from pepper (*Capsicum annuum* L.) [46] and *Chrysanthemum lavandulifolium* [47].

Transcription factors (TFs) are important regulators that control gene expression in the plant body and play important roles in cell signaling transduction and stress responses [48]. NLPs are essential TFs involved in nitrate sensing/response, symbiosis, and abiotic stress [49–51]. Predicted subcellular localization results showed that the *AhNLPs* genes were localized in the nucleus or/and chloroplasts (Table 1). In this study, the subcellular localization patterns of *AhNINa* and *AhNINb* were analyzed. *AhNINa* and *AhNINb* were located in the cell nucleus (Figure 6), demonstrating that they are nuclear-localized transcription factors.

Unlike most other plants, legumes can form root nodules in association with rhizobia, which can fix atmospheric N₂ into ammonia [52–56]. In peanut, transcriptomic analysis showed that *AhNLP2e* was widely expressed in all tested tissues including the embryo, testa, and root nodules (Figure 4). In addition, *AhNLP4a* and *AhNLP4b* were highly expressed in the root tip and root nodule (Figure 4), which suggests potential functional redundancy and co-regulation. Moreover, the transactivation assay proved that *AhNINa* and *AhNINb* act as a transcriptional activator (Figure 7), indicating that they may play a positive role in peanut nodule formation. Interestingly, phylogenomic analyses revealed that the transcription factor NIN is essential for nodulation in nitrogen-fixation-clade species [57,58]. In *L. japonicus* and *P. sativum*, the expressions of *LjNIN* and *PsNIN* were detected in young and mature nodules, and the loss of the single *LjNIN* or *PsNIN* function leads to abolished nodule initiation [7,10]. Consistent with *L. japonicus* and *P. sativum*, *AhNINa* and *AhNINb* are also expressed in root nodules (Figures 4 and 5A). Furthermore, the promoter-GUS staining analysis confirmed the nodule-specific expression patterns of *AhNINa* and *AhNINb* (Figure 5B–E). It was reported that *AhNINa* and *AhNINb* genes are expressed in early developmental stage nodules, such as 1, 3, and 4 dpi nodules, and play important roles during nodule initiation [59]. In this study, we found that *AhNINa* and *AhNINb* genes are also expressed in developing nodules, such as 7 and 14 dpi nodules. In soybean (*Glycine max*), there are four putative orthologous NIN genes, *GmNIN1a*, *GmNIN1b*, *GmNIN2a*, and *GmNIN2b*, and the *Gmnin1a nin1b nin2a nin2b* quadruple mutant displayed defects in root nodule formation [8]. However, there is no genetic evidence that *AhNINa* and *AhNINb* genes are involved in peanut nodule formation. In this study, the down-regulation of the expression of both *AhNINa* and *AhNINb* by RNAi also results in peanut defects in nodule formation (Figure 8), demonstrating that *AhNINa* and *AhNINb* genes play vital roles in the nodule development of peanut. Our work provides insight into the understanding of the redundant function of *AhNINa* and *AhNINb* genes in peanut nodule development.

5. Conclusions

This study provides the genome-wide characterization of the NLP family members in peanuts. A total of 16 NLP genes were identified, including 2 *AhNIN* and 14 *AhNLP*, and their chromosome locations, phylogenetic relationship, and gene function, especially the

function of *AhNINa* and *AhNINb* genes, were analyzed. Both *AhNINa* and *AhNINb* genes are specifically expressed in root nodules and the silencing of *AhNINa* and *AhNINb* by RNA interference (RNAi) led to the failure of nodule formation. Future work should focus on verifying whether increasing the expression of *AhNINa* and *AhNINb* can produce more nodules and incorporate them into breeding application strategies to improve nitrogen fixation efficiency.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy15102377/s1>, Figure S1: Chromosome distribution of *AhNIN* and *AhNLP* genes in *A. hypogaea*. Figure S2: Sequences of the ten identified motifs in *AhNIN* and *AhNLP* proteins. Figure S3: Multiple sequences alignment of the *AhNIN* and *AhNLP* protein members. Figure S4: *AhNINa* and *AhNINb* gene CDS alignment. Table S1: Primers used in this study. Data S1: Protein sequences of MtNIN, MtNLP, AtNLP, AhNLP, and AhNIN used to run the BLAST analysis and used to construct the phylogenetic tree.

Author Contributions: Conceptualization, H.W. (Hongfeng Wang); methodology, Y.R., L.W. and F.Z.; software, G.C., Z.L., Y.T. and Y.W.; investigation, H.W. (Hongfeng Wang), Y.X. and H.W. (Hongwei Wang); writing—original draft preparation, H.W. (Hongfeng Wang) and H.W. (Hongwei Wang); writing—review and editing, H.W. (Hongfeng Wang) and M.Y.; supervision, H.W. (Hongfeng Wang) and M.Y. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

NIN	NODULE INCEPTION
NRE	Nitrate-responsive <i>cis</i> -element
ANR1	ARABIDOPSIS NITRATE REGULATED 1
NRT2	NITRATE TRANSPORTER 2
LBD37	LATERAL ORGAN BOUNDARY DOMAIN 37
AtTCP20	TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1-20
CYP707A2	CYTOCHROME P450 MONOOXYGENASE 707A2
NFR5	NOD FACTOR RECEPTOR 5
CCaMK	Ca ²⁺ /CALMODULIN DEPENDENT KINASE
HK1	HISTIDINE KINASE 1
NSP2	NODULATION SIGNALING PATHWAY 2
NIR	NITRITE REDUCTASE
RNAi	RNA interference

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