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Genome-Wide Identification, Characterization of the *NIN-Like Protein* Gene Family in *Phoebe bournei* and Its Heat-Responsive Expression Patterns

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Abstract

NIN-like proteins (NLPs) serve as transcription factors specific to plants that serve a central function in signaling and stress response in terms of nitrogen; however, their contribution to heat tolerance in woody plants remains poorly understood. Here, we identified 6 *PbNLP* genes in *Phoebe bournei* (Hemsl.) Yen C. Yang, an endangered tree species, and characterized their evolutionary relationships as well as gene and promoter elements, predicted protein structures and interactions, functional enrichment, tissue-expression profiles, and heat-responsive dynamic expression patterns with physiological indicators. The 6 genes were distributed among three of the four phylogenetic groups, and segmental duplication appeared to be the principal driver of family expansion. All PbNLP proteins possessed intact RWP-RK and PB1 domains, and their promoters were enriched in stress and hormone-responsive cis-elements. Secondary and tertiary structure predictions indicated a predominance of random coils, an absence of predicted beta-turns, and a conserved architecture comprising a compact core and flexible flanking regions. Protein interaction analysis predicted associations with several regulatory partners, particularly ROPGEF14, with PbNLP3 showing the highest network connectivity. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment showed the *PbNLP* family Complete stress responses via hormone-signaling pathways. Transcript abundance was generally higher in leaves and bark than in xylem. Importantly, under heat stress, *PbNLP* genes showed expression divergence and most of them were positively correlated with one another, while differing substantially in their temporal expression trends, which were divided into two major response types. The *PbNLP3* showed negative correlation with other genes and higest expression level. Analysis of heatmap revealed *PbNLP5* exhibited the strongest correlations with photosynthetic physiological traits. Collectively, these findings identify *PbNLP3* and *PbNLP5* as candidate gene for further investigation of a potential crosstalk between nitrogen signaling and heat stress responses in *P. bournei* and provide potential targets for breeding heat-tolerant trees.

Keywords *Phoebe bournei*; NLP transcription factor; tree thermotolerance; gene expression pattern; physiological adaptation; molecular breeding

Introduction

Nitrogen (N) signaling transduction serves an essential function in the central regulatory network of plants to control nutritional state and the responses to environmental challenges (Castaings et al. 2009; Konishi and Yanagisawa 2013). NLPs act as plant specific transcription factors that transduce

nitrate signals into transcriptional reprogramming via their RWP-RK DNA-binding domain and PB1 protein interaction domain: RWP-RK DNA-binding domain and PB1 protein interacting domain (Konishi and Yanagisawa 2013). The conserved RWP-RK and PB1 domains are indispensable for NLP proteins to execute their biological functions in nitrate sensing and signal transduction (Mu and Luo 2019). In *Arabidopsis thaliana*, NLPs are major regulators of nitrate responses, and directly regulate more than 1000 target genes by post-translational modification (Konishi and Yanagisawa 2013). Genome-wide identification of *NLP* family has been performed in moss *Physcomitrella patens*, revealing their potential function in nitrogen response (Jan et al. 2023). New findings have expanded the roles of NLPs to not only control of nitrogen signaling but also environmental stress responses such as cold stress response and abscisic acid (ABA) signaling pathway regulation (Ding et al. 2022; Huang et al. 2025). Bioinformatic studies of *NLP* genes in rice and other herbaceous plant species have shown that they have numerous stress responsive cis-acting elements within their promoter regions, including MYB-binding sites, indicating that NLP expression is regulated by environmental stimuli (Konishi and Yanagisawa 2013; Liu et al. 2017; Liu et al. 2018; Jagadhesan et al. 2020). Beyond that, the responsiveness of NLP proteins to environmental change might be controlled via post-translational manipulation particularly phosphorylation by CPK (Liu et al. 2017; Ding et al. 2022). NLP7 and NLP8 are part of one of two regulatory hubs of *A. thaliana*, integrating nitrogen and stress stimuli with ABA and calcium-sensitive protein kinase pathways (Ding et al. 2022; Huang et al. 2025). Considering all these characteristics, it is possible that NLPs can coordinate thermotolerance by altering nitrogen distribution and antioxidant defense. Current reviews have already presented the current research advances of NLPs in herbaceous plants (Sámano et al. 2024), but the regulatory processes of NLPs in woody plants remain unknown.

Phoebe bournei (Hemsl.) Yen C. Yang is classified as a rare and endangered woody species on the national level in China and due to its high-quality timber, it is considered to have a considerable economic value (Li et al. 2018; Wu et al. 2022). Nonetheless, extreme temperatures, caused by greenhouse effect (40°C) affect the photosynthesis system negatively, the nitrogen use efficiency (NUE) decreases, and regeneration does not occur (Xu et al. 2022). Heat stress interrupts nitrogen assimilation and recycling of forest trees, leading to greater inhibition of growth and poor wood quality (Balducci et al. 2016; Zhao et al. 2022; Liao et al. 2024; Chen et al. 2025). *P. bournei* growth responses to heat stress occurs in three major pathways which comprises the breakdown of thylakoid membrane

structures with reduction of the photochemical efficiency, excess creation of reactive oxygen species (ROS) as well as abnormal nitrogen assimilation process in photosynthetic tissues (Lin et al. 2025). As of today, it has been established that transcription factor family DBB, CORBA, bZIP, and HSFs are important regulatory factors of both the responses to abiotic stress and heat responses in *P.bournei* (Guo et al. 2024; Liao et al. 2024; Cao et al. 2026; Ying et al. 2026). These studies provied a solid fundation of our research. However, the function of nitrogen signal regulators during stress responses in *P. bournei* remains unknown. Since the NLPs are conserved across evolution, as principal regulators of nitrate transporter genes in herbaceous plants (Castaings et al. 2009; Liu et al. 2017), and they may be stress responsive (Konishi and Yanagisawa 2013; Liu et al. 2017; Ding et al. 2022; Huang et al. 2025; Li et al. 2025), it could be speculated that the *PbNLP* gene family might participate in heat stress response regulation in *P. bournei* through modulation of nitrogen metabolism pathway.

Nevertheless, several critical questions remain unanswered regarding the *NLP* gene family in woody species like *P.bournei*. First, it is unclear whether the evolutionary trajectory and expansion mechanism of *NLPs* in this endangered timber tree differ from those in herbaceous plants, given its distinctive secondary growth and nitrogen storage strategies. Second, the tissue-specific expression landscape of *PbNLP* genes, particularly whether they exhibit woody-specific features, has not been characterized. Third, the temporal dynamics of *PbNLP* expression under progressive heat stress and the identity of the key regulatory members remain elusive. Addressing these questions is essential not only for understanding the regulatory evolution of NLPs in woody perennials but also for identifying potential targets for breeding heat-tolerant *P. bournei* in the context of global warming. To address such gaps in the knowledge we have selected the *PbNLP* gene family on a genome-wide basis and analyzed them using multi-dimensional methods, such as phylogenetic analysis, syntenic analysis, promoter analysis, and expression patterns. In particular, we studied the evolution and duplication of *PbNLP* genes, expression profiles in selected tissues, functional enrichment, temporal changes of the expressions of the *PbNLPs* under sustainable heat stress with physiological analysis. Our study would give certain background genetic insight into the clarification of the regulation mechanisms of the *PbNLP* gene family and present target genes with hypothesis to elucidate how to increase heat stress tolerance in forest trees and woody species.

Materials and methods

The Treatment of Plant Materials

The chosen *P. bournei* seedlings had been both mature and similar in size since they were conditioned pre-experimentally in a temperature-controlled incubator prior to the application of the stress treatment. Before subjecting them to heat stress treatment, the roots were washed in distilled water and dried with a piece of absorbent paper. Heat stress treatment was initiated at 8:30 a.m. Leaf samples of *P. bournei* were sampled at 6 time points: 0 (Ck), 1, 3, 6, 12, and 24 hours. At each time point, three groups were tested with three seedlings per group (pooled to form a single biological replicate). All samples were stored for subsequent experiments after collection (Lin et al. 2025).

Identification of *PbNLP* Gene Family and Physicochemical Characterization Analysis

The whole protein sequences, genomic sequences, and GFF3 files of *P. bournei* were retrieved from the China National GeneBank Database (https://db.cngb.org/search/project/CNP0002030/) (Chang et al. 2023). The protein sequences of *A. thaliana Nin-like* genes were acquired from The *Arabidopsis* Information Resource (TAIR) (https://www.arabidopsis.org).

Using *A. thaliana Nin-like* genes as the reference, the *NLP* conserved domain HMM profile (PF00564) was obtained at the Pfam database and the HMMsearch program with an E-value of less than 1e-5 was used to find the protein sequences of *P. bournei*. The second retrieval was performed via Blast method, and redundant sequences from the two retrievals were removed. The intersection of the two retrieval results was obtained, and the difference set sequences were verified for conserved domains using Pfamscan (https://www.ebi.ac.uk/Tools/pfa/pfamscan/) and the NCBI Batch CD-search tool. Sequences containing NLP conserved domains were screened to identify the *PbNLP* gene family (Konishi and Yanagisawa 2013; Liu et al. 2017; Li et al. 2025). The genes were renamed according to their chromosomal localization and arrangement order.

After the identification and renaming, we do the physicochemical characterization and subcellular localization. We employed ExPASy ProtParam to analyze the physicochemical characterization of PbNLP proteins(https://web.expasy.org/protparam/) (Gasteiger 2003). We also predicted the subcellular localization of PbNLP proteins was predicted using WoLF PSORT (https://wolfpsort.hgc.jp/) (Horton et al. 2007).

Finally, the conserved functional domains of *P. bournei* PbNLP protein sequences were verified using the batch search tool for conserved domains and CD-search page of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The position-specific score matrix (PSSM)

IDs corresponding to each sequence were screened and extracted to complete the gene alias annotation (Marchler-Bauer et al. 2011).

Chromosomal Localization

Using the *P. bournei* genome annotation file in the GFF3 format, data on the chromosome of *PbNLP* genes was derived and the output of chromosomal location of the given gene family was evaluated with the assistance of TBtools software (Chen et al. 2020).

Gene Structure, Conserved Domain, and Motif Analysis

The whole genome sequences and GFF3 files of the *PbNLP* genes were filtered to obtain the DNA and coding sequences of *PbNLP* genes. The distribution properties of exons, introns, and untranslated regions (UTRs) were evaluated through Gene Structure Display Server (GSDS, http://gsds.gao-lab.org/index.php) (Hu et al. 2015). We used NCBI Batch CD-search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) to evaluate the conserved domains of PbNLP proteins (Marchler-Bauer et al. 2011). The analysis of conserved motifs of PbNLP proteins was performed by using MEME 5.3.3 (https://meme-suite.org/meme/) setting the maximum value of conserved motifs at 5. Conserved domains and motif analysis results were illustrated using TBtools software (Bailey et al. 2015; Chen et al. 2020).

Promoter Cis-Acting Elements Analysis

The application of TBtools was used to derive a nucleotide sequence of 2000 base pairs upstream of the start codon of the *PbNLPs* coding sequences. Prediction of cis-acting elements Prediction of cis-acting elements Prediction of cis-acting elements was done by using the PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Chen et al. 2020). Microsoft Excel was used to process the data and it was visualised in the TBtools software to examine the nature of the distribution of cis-acting elements (Chen et al. 2020).

Phylogenetic Tree Construction and Synteny Analysis

The *Solanum lycopersicum Nin-like* gene sequences were obtained by an online search on the Solanaceae Genomics Network (http://solgenomics.net/). The outcomes obtained were the NLP protein sequences of *P. bournei* and *A. thaliana* as previously stated. NLP protein sequences of *P. bournei*, *A. thaliana* and *S. lycopersicum* were aligned multiple time using Clustal W of the MEGA12.0 program with the default settings. The Neighbor-joining (NJ) method was adopted to construct a phylogenetic

tree with 1000 bootstrap replicates to explain the phylogenetic relationship between *NLP* genes in all the three species (Saitou and Nei 1987).

The Genome and GFF files of *Populus trichocarpa*, *Oryza sativa*, *A. thaliana*, and *Zea may* were retrieved from the National Center of Biotechnology Information (NCBI). Annotation of genes and genomic data for *P. bournei* were similar to those mentioned previously. The intragenomic synteny of *P. bournei*, inter-species synteny between *P. bournei* and *A. thaliana*, *P. bournei* and *O. sativa*, *P. bournei* and *P. trichocarpa*, and *P. bournei* and *Z. may* were analyzed with using the one-click analysis tool of MCScanX in TBtools software. The synteny map was plotted with the help of the super-fast plotting plug-in of TBtools and the core genes were highlighted in bold (Chen et al. 2020).

The Protein Structure Prediction

The SOPMA online server was employed to have a predict of *the PbNLP* gene family's secondary protein structure (Geourjon and Deléage 1995), and the proportion of alpha helices, beta turns, random coils , and extended strands for each PbNLP protein were predicted. For predicting the three-dimensional conformations of PbNLP proteins, homology modeling was conducted via the SWISS MODEL server (https://swissmodel.expasy.org). Searching for templates for the first time, we chose the highest coverage one to build the model for our consequence of the *PbNLP* gene family's tertiary protein structure.

Prediction and Visualization of PPI Network

According to STRING database (https://string-db.org), we build a protein-protein interaction (PPI) network to predict the connection between the PbNLP protein and the AtNLP protein (Szklarczyk et al. 2019). Our limit of interaction score is not higher than 10 with high confidence level parameter (0.7) and unconnected nodes are removed in the network. The Cytoscape software was utilized to beautify the network consequence.

GO and KEGG Enrichment Analysis

The eggNOG-mapper3 was used to get the annotation sequences by putting in the whole protein sequences of *P. bournei* (Cantalapiedra et al. 2021; Hernández-Plaza et al. 2026). After getting the sequences, the Tbtools was used to complete the GO and KEGG enrichment analysis. The results was visualized by R package (Yu et al. 2012).

Analysis of Tissue-Specific Expression Profiles

The profile of *PbNLP* gene expressions in different tissues of *P. bournei* was retrieved in the BioProject database (https://ngdc.cncb.ac.cn/bioproject/) which involves root bark, root xylem, stem bark, and stem xylem as well as young leaves (Accession nuber: PRJNA628065). In order to analyze tissue-specific expression of *PbNLP* genes, TBtools software was used to normalize expression profile data to compare the normalized expression heatmap (Chen et al. 2020).

Heat Treatment-Induced Expression and Correlation Heatmap Analysist

Based on the *Phoebe bournei* Genome Database (https://pbgenome.org.cn), we utilized the gene expression and correlation heatmap function under the transcriptomics module to complete the analysis. We select the heat stress dataset with the Pearson analysis under the log2 transform, with the FPKM data format, then generate a heatmap and download the matrix. The last procedure is to plot it and have our consequence look good with TBtools (Chen et al. 2020).

Analysis of Quantitative Real-Time PCR (qRT-PCR)

Total RNA extraction in leaf samples under high-temperature stress treatment was conducted using the HiPure Plant RNA Mini Kit (Magen, Shanghai, China). First cDNA synthesis was performed with PrimeScript RT reagent kit (Perfect Real Time, Takara, Dalian, China). The specific primers used to amplify *PbNLP* genes were designed on the basis of non-conserved regions of *PbNLP* genes using the Primer 3.0 software and synthesized by Fuzhou Qingbaiwang Biotechnology Co., Ltd. (Fuzhou, China).

The PCR system (20 ul) contained 1ul of cDNA template, 10ul of SYBR Premix Ex Taq TM II, 2ul of specific primers, and 7ul of nuclease free water. The amplification profile was: first denaturation at 95 ℃ for 30s, followed by 40 cycles of 95 ℃ (5s), and 60 ℃ (30s) for denaturation and annealing/extension, respectively, then 95 ℃ (5s), 60 ℃ (60s) and finally incubation at 50 ℃ (30s). PbEF1α (GenBank Accession No. KX682032) served as the internal reference gene. Relative transcript levels of *PbNLP* genes were calculated via the $2^{-\Delta\Delta Ct}$ formula (Li et al. 2025). Three biological replicates were used. Normality was tested using the Shapiro-Wilk test, and one-way ANOVA followed by Tukey's HSD test for multiple comparisons was used to test and visualize the significance between Ck group and other groups (Jan and Shieh 2014).

Visualization of Photosynthetic Physiological Indicators of the *PbNLP* Gene Family

Since the gene expression and photosynthetic physiological indicator data deviated from a normal distribution, Spearman’s rank correlation analysis was applied to assess the association between them

(Hotelling and Pabst 1936). The genes and physiological indicators correlation heatmap was depicted using the TBtools software (Chen et al. 2020).

Results

Identification of the *PbNLP* Gene Family and Its Physicochemical Characterization

In the present study, a total of 6 members of the *PbNLP* gene family were found. They all have been renamed *PbNLP1-PbNLP6*. The studies regarding the physicochemical properties of PbNLP proteins showed great variation in amino acid length and molecular weight between the family members: the number of amino acids was found to be between 844 and 1016 and their relative molecular weights were 94.3 kDa-111.9 kDa (Table 1).

Analysis of the theoretical isoelectric point (pI) showed that pI values for PbNLP proteins ranged from 5.29 to 8.26: five members (PbNLP2 to PbNLP6) were acidic (pI < 7), and only PbNLP1 was a basic protein (pI = 8.26). Such charge heterogeneity might influence the solubility of the proteins at physiological pH and their binding to other biological macromolecules. Instability index prediction showed that all PbNLP proteins had instability indices greater than 40 (48.63-58.03), which makes them unstable proteins. The aliphatic index was between 74.99 and 79.52, which means that the proteins have a moderate thermostability. Hydropathicity scores grand average ranged between -0.408 and -0.349, and all the scores were negative, suggesting that PbNLP proteins are generally hydrophilic, which helps them remain in a solution state in the cellular aqueous medium. Assays of subcellular localization predicted that all 6 PbNLP proteins are located in the cell nucleus. This discovery also implies that they could be transcription factors, an evolutionarily conserved characteristic of the *NLP* gene family.

Table 1 Physicochemical properties of *PbNLP* genes in *Phoebe bournei*.

Gene Name	Gene ID	Number of Amino Acid	Molecular Weight	Theoretical pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity	Subcellular Location
PbNLP1	*OF25772*	844	94311.21	8.26	48.63	78.42	-0.408	Nucleus
PbNLP2	*OF08057*	872	97195.23	6.01	50.75	74.99	-0.418	Nucleus
PbNLP3	*OF01171*	959	105483.63	5.84	48.49	75.31	-0.375	Nucleus

PbNLP4	*OF01036*	957	105319.93	6.31	53.07	79.52	-0.36	Nucleus
PbNLP5	*OF24652*	884	97566.36	5.34	56.52	75.35	-0.349	Nucleus
PbNLP6	*OF09021*	1016	111947.3	5.29	58.03	77.32	-0.373	Nucleus

Phylogenetic Reconstruction and Evolutionary Assessment

Phylogenetic trees were produced by the neighbour-joining approach using the protein sequences of NLPs from *P. bournei*, *A. thaliana* ,and *S. lycopersicum* to examine the phylogeny of the *PbNLP* genes (Fig. 1). It has been established that the *PbNLP* gene family can be separated into four different subgroups (A-D).

6 *PbNLP* genes were unevenly distributed across three subgroups (Groups A, B, and D), and no *P. bournei* homologous genes were detected in Group C. Besides, Group D contained the most members, including three *P. bournei* genes (*PbNLP1, PbNLP2*, *PbNLP4*) and multiple homologous genes from *A. thaliana* and *S. lycopersicum*. Group B included *PbNLP5* and *PbNLP6*, which clustered closely in the phylogenetic tree and formed a homologous subclade with *S. lycopersicum* genes such as *SlNLP5* and *SlNLP6*. Notably, Group A contained only one *P. bournei* gene (*PbNLP3*), which clustered with *AtNLP8*, *AtNLP9* and *SlNLP4*, *SlNLP6*, forming a mixed cluster.

In most cases, it is assumed that all *PbNLP* genes fall into the same subfamily with their homologs in *A. thaliana* and *S. lycopersicum*, suggesting that *PbNLP* gene family has been preserved during the course of evolution in dicotyledonous plants.

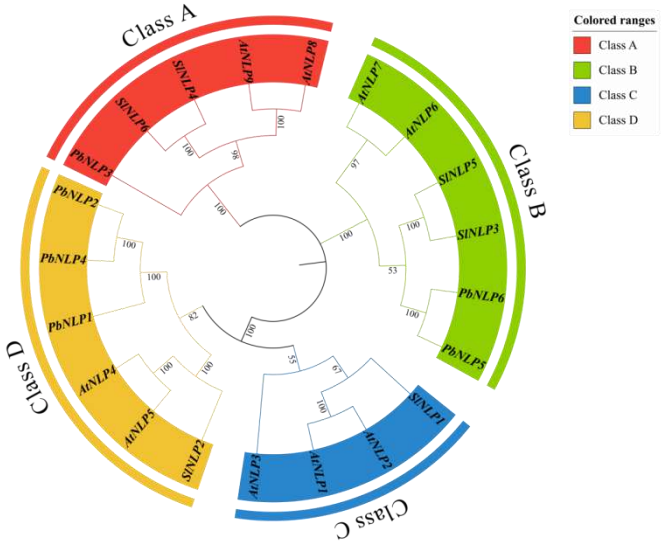


Fig. 1 Phylogenetic relationships of NLP proteins. The neighbor-joining (NJ) phylogenetic tree was built from NLP amino-acid sequences of *Phoebe bournei* (Pb), *Arabidopsis thaliana* (At) and *Solanum lycopersicum* (Sl).

Chromosomal Localization Analysis

Based on the whole-genome annotation information, chromosomal localization analysis placed the 6 *PbNLP* genes on 6 different chromosomes (Chr03, Chr04, Chr05, Chr06, Chr07, and Chr12), each on a separate chromosome, with no tandem duplicated gene clusters observed (Fig. 2). In terms of chromosomal positions, the *PbNLP* genes showed obvious regional preference. Among them, 4 genes (*PbNLP2*, *PbNLP3*, *PbNLP4*, *PbNLP6*) were concentrated in the upper regions of chromosomes (approximately 10 to 20 Mb), accounting for 66.7% of the total family members. Notably, *PbNLP5* was located in the terminal region of Chr07 (about 70 Mb), close to the telomere. By comparison, *PbNLP1* was solely localized in the middle of Chr03 (about 45 Mb) and such a mid-regional localization could confer on it a more constant expression pattern and different regulation. When chromosome size was considered, the position distribution of *PbNLP* genes did not have any significant correlation with chromosome length. The longest and shortest chromosomes each contained a solitary *PbNLP* gene, and there was no trend toward larger chromosomes having more *PbNLP* genes. The longest chromosome (Chr03, about 110 Mb) carried *PbNLP1*, while the shortest chromosome (Chr12, about 55 Mb) carried *PbNLP6*, with similar gene densities between the two.

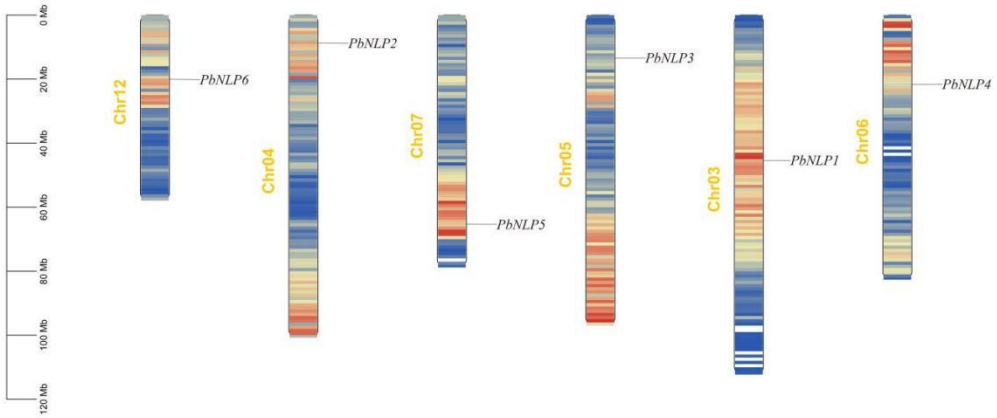


Fig. 2 Chromosomal localization of *PbNLP* genes in *Phoebe bournei*. The haploid chromosome number of *P.bournei* is 12, with the scale unit in Mb.

Intraspecific Synteny Examination of the *PbNLP* Gene Family

Intraspecific synteny analysis showed the specific distribution of *PbNLP* genes on each chromosome of the *P. bournei* genome. Every chromosome was identified by gene localization and different colors were used to represent the different types of duplications such as tandem duplication, segmental duplication, and syntenic mechanism (Fig. 3).

Analysis of gene duplication events revealed a unique pattern consisting of two independent syntenic modules and one isolated gene. Module A was composed of Chr03, Chr04, and Chr06, forming a tight triangular network connection: Chr03 (*PbNLP1*) was directly syntenically linked to Chr04 (*PbNLP2*), and both were syntenically related to Chr06 (*PbNLP4*). Such network structure indicates that *PbNLP1*, *PbNLP2*, and *PbNLP4* may have arisen from an ancient whole-genome duplication (WGD) event, and Chr03 and Chr04 preserve the most intact ancestral block structure as twin cores as suggested by the absence of collinear signals. Notably, although Chr11 was syntenic with Chr03 and Chr04, it did not carry any *PbNLP* genes, which may represent a gene loss event of this family during evolution. Module B contained only Chr07 (*PbNLP5*) and Chr12 (*PbNLP6*), with a single syntenic link between them and complete isolation from Module A. This independent duplication unit implies that *PbNLP5* and *PbNLP6* may have undergone independent duplication events or separated from Module A during genome rearrangement, as suggested by the absence of collinear signals between Module B and Module A. The most distinctive was *PbNLP3* (located on Chr05), whose chromosome showed no syntenic connections and was completely isolated from the above two modules. Combined with the result of phylogenetic analysis that *PbNLP3* clustered

independently in Group A, it is tentatively proposed that this gene might have originated via transposon-mediated duplication, transposition, or independent duplication events and has since diverged significantly in its sequences leading to the disappearance of syntenic signals, although this inference requires validation with transposon annotation.

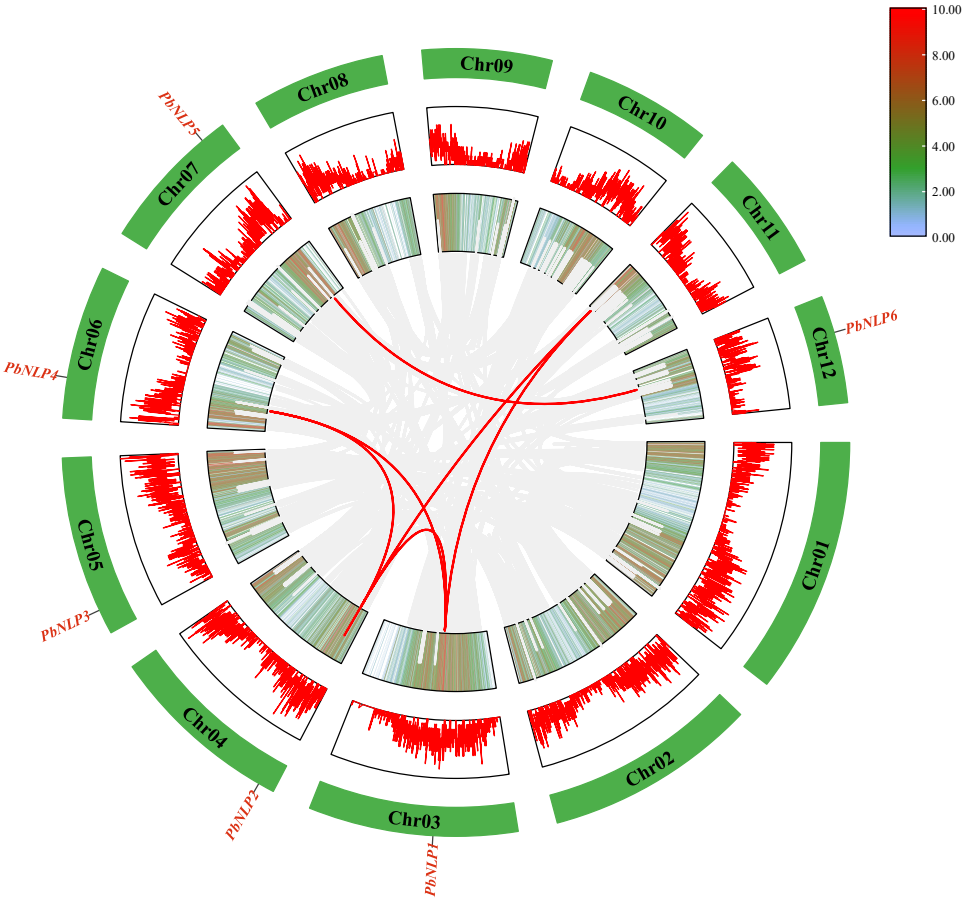


Fig. 3 Genomic physical locations, duplication events, and syntenic relationships of *PbNLP* genes in *Phoebe bournei.* The localization of genes on each chromosome is easily indicated, and different duplication events (such as tandem duplication and segmental duplication) and syntenic processes are indicated by colored lines.

Interspecific Synteny Analysis of the *PbNLP* Gene Family

In order to investigate the evolutionary conservation and divergence among *PbNLP* family members, we performed interspecies synteny comparison with *P. bournei* and four model plants (Fig. 4). The chosen taxa included both dicot and monocot lines, as well as herbaceous and woody plants, thus enabling to thoroughly examine the evolutionary conservation of *PbNLP* genes.

All four comparative genomes had collinear orthologous *NLP* gene pairs with significant variations in the quantity of synteny being recorded. The largest number of syntenic partners (16 pairs) was provided by *P. trichocarpa*, which is significantly higher than the number of syntenic partners of

the three herbaceous species (*A. thaliana*, *O. sativa* and *Z. may*, with 11, 9 and 11 pairs respectively). In contrast, the least number of collinear matches was seen in *O. sativa*. This uneven distribution of syntenic loci indicated that *NLP* genes had a higher level of genomic conservation between the two woody plants.

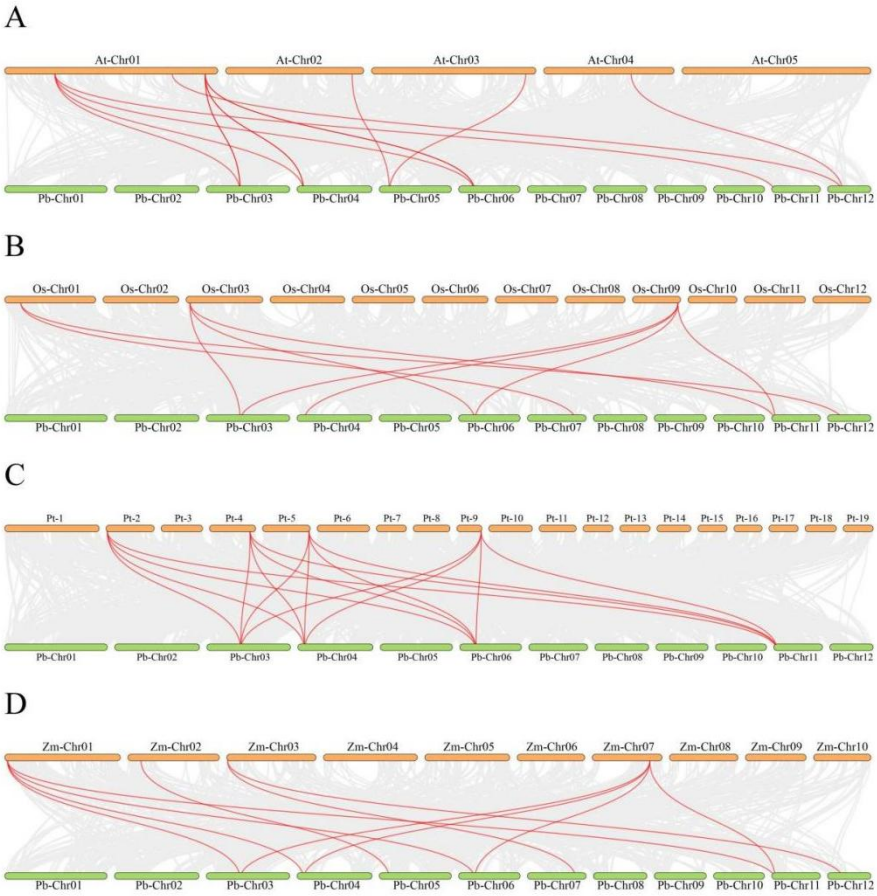


Fig. 4 Interspecific synteny analysis of *NLP* genes between *Phoebe bournei* and three model plants. **(a)** *P. bournei–A. thaliana* synteny map; **(b)** *P. bournei–O. sativa* synteny map; **(c)** *P. bournei– P.trichocarpa* synteny map; **(d)** *P. bournei–Z.may* synteny map.

Conserved Domains, Motif Composition, and Gene Structure Analysis

In order to investigate the structural conservation and differentiation properties of the PbNLP family in *P.bournei*, we examined its conserved domains, motif composition, and gene structure in detail, as it represents a core structure of the further research of genes specific expression in different tissues and responses to heat stress (Fig. 5). Analysis of conserved domains indicated that each of the 6 PbNLP proteins has intact RWP-RK domains (PF02042) and PB1 domains (PF00564), with no deletions or shifts. The RWP-RK domain is responsible in DNA binding whereas the PB1 domain helps in protein-protein interactions and both sequences and layouts are highly conserved indicating that the

family has retained entirely the central transcription factor functional framework of the NLP family. Conserved element screening revealed 8 conserved motifs (Motif 1-8). The numbers, types, and arrangement of motifs among all members of the family were identical and had no clade-specific variations. Of them, Motif 2 was fully consistent with the RWP-RK domain region, which acts as the main DNA-binding sequence; the rest of the motifs are mostly engaged in the maintenance of protein structure stability. There were no characteristic motifs found in PB1 domain, which can be present in a specialized form, due to the functional conservation and redundancy of this gene family. These genes have been structurally examined to show that the *PbNLP* family has 5 to 6 exons with regular intron distribution, which generally displays the properties of core conservation and local diversification. *PbNLP1*, *PbNLP3*, *PbNLP4* and *PbNLP6* have intact 5 and 3 ends of the 5 and 3 untranslated regions (UTRs) and complex exons, with normal transcriptional regulation patterns; *PbNLP2* has only 3 end UTRs, with a much simpler structure; *PbNLP5* has no UTR regions at all, implying a very specialized gene architecture. The sizes of these loci vary within the range of 6500 bp to 18500 bp (*PbNLP4* is the largest and *PbNLP6* is the smallest). Integrity of UTR and the length of genes provide various potentials of transcriptional responses to the family: the streamlined structure of *PbNLP2* could allow quick transcription initiation whereas the specialized structure of *PbNLP5* accommodates the precise regulatory demands during stress. This forms an important structural foundation of the future dissection of the functional differentiation of the family in nitrogen metabolism homeostasis and reaction to heat and drought stresses.

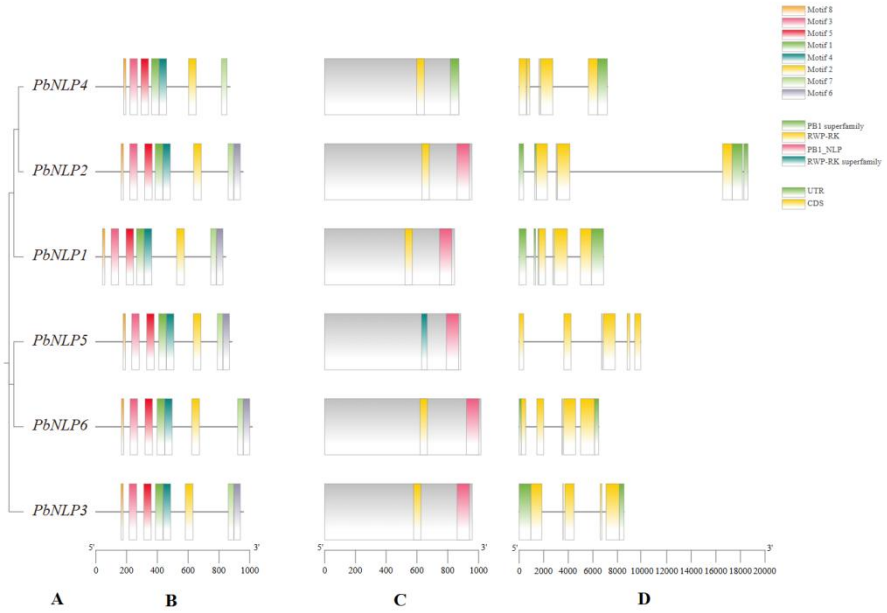


Fig. 5 Combined analysis of phylogeny, conserved motif distribution, conserved domain architecture and exon-intron organization for the *PbNLP* gene family. **(a)** Phylogenetic tree of the *PbNLP* gene family. **(b)** Conserved motifs of PbNLP proteins. The 8 conserved motifs are represented by rectangles with different colors and numbers. **(c)** Conserved domains of PbNLP proteins. **(d)** Schematic diagram of *PbNLP* gene structures. Green rectangles represent coding sequences (CDS) and yellow rectangles represent untranslated regions (UTR), respectively. Black lines indicate introns.

Promoters Cis-Acting Elements Analysis

To elucidate the regulatory processes that regulate the expression and biological roles of *PbNLP* genes, their promoter sequence of each *PbNLP* gene has been analyzed and 19 cis-acting elements have been identified (Fig. 6). Through the consequence, we found that each *PbNLP* gene has an abscisic acid responsiveness element and an anaerobic induction element. Some *PbNLP* genes, like *PbNLP1*, *PbNLP2*, *PbNLP4*, *PbNLP5,* and *PbNLP6*, have a gibberellin-responsive element, while *PbNLP1* has the MeJA-responsiveness element. The elements mentioned above all relate to the heat tolerance of plants, so we can infer that *NLP* genes play a key role in regulating *P.bournei*'s heat tolerance ability.

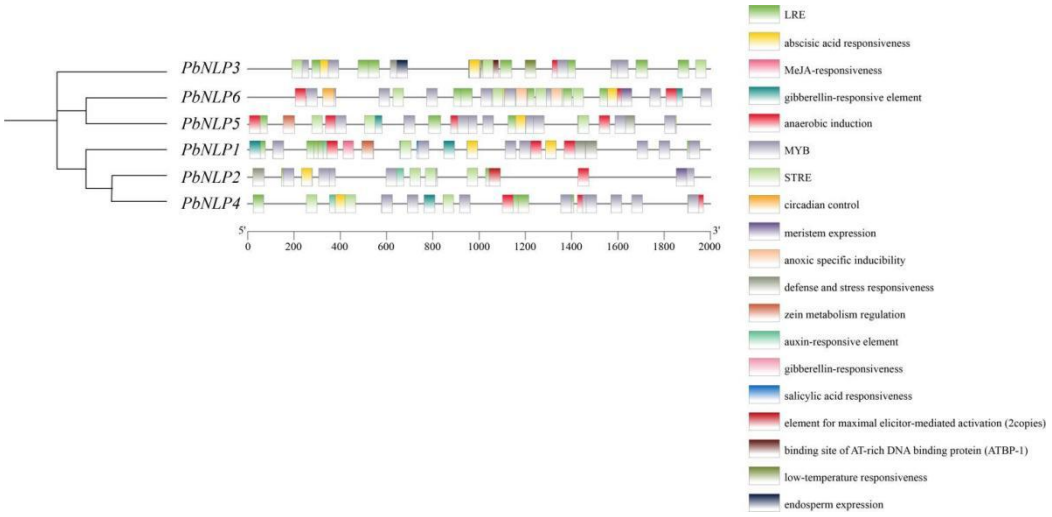


Fig. 6 Distribution characteristics of cis-acting elements in the promoters of the *PbNLP* gene family in *Phoebe bournei.* The left side shows the phylogenetic tree of the *PbNLP* gene family, and the right side shows the distribution of cis-acting elements in the 2000 bp promoter region upstream of the ATG of each gene; different colors represent different element types, rectangles represent independent element sites, and the horizontal axis represents the physical position (bp) of the promoter region, intuitively displaying the distribution and abundance characteristics of elements.

The Structure Prediction of PbNLP Family

To analyze the conformational features of PbNLP proteins, the secondary structure proportions of 6 PbNLPs were predicted and quantified, and a tertiary structure prediction model was constructed. Secondary structure statistics showed that random coils accounted for the highest proportion (63.98%-71.75%), followed by α-helices (20.18%-25.36%), and extended strands the lowest (7.34%-10.66%). It is important to note that β-turns were not present at all in any PbNLP proteins (Table 2). The absence of locally compacted bent structures in the polypeptide chains indicates that the overall conformation is relatively extended and flexible, indicating that PbNLP interactions with other proteins might not depend on β-turns but the α-helices and random coil regions. Of the members, PbNLP1 has the greatest percentage of both α-helix and extended strand, whereas PbNLP6 has the least α-helix content and the highest percentage of random coils. The prediction of tertiary structure also indicated that all PbNLP proteins have a compact core and flexible disordered flanks architecture (Fig. 7). In such a model, α-helical and a few long strands form a tight spherical core, and large unstructured regions form flexible ribbon-like flanks, an arrangement that maintains core functionality but allows flexibility of conformation. Although all PbNLP members share a similar overall backbone, subtle differences in the length and spatial extension of the flexible disordered regions are observed, which correlate well with the secondary structure composition of each member.

Table 2 In silico prediction of secondary structure elements in PbNLP proteins.

protein	Proportions of Secondary-Structure Elements(%)			
	α-helix	**β-helix**	**Random Coil**	**Extended Strand**
PbNLP1	25.36	0.00	63.98	10.66
PbNLP2	22.48	0.00	70.18	7.34
PbNLP3	24.19	0.00	66.94	8.86
PbNLP4	22.99	0.00	69.59	7.42
PbNLP5	23.98	0.00	65.95	10.07
PbNLP6	20.18	0.00	71.75	8.07

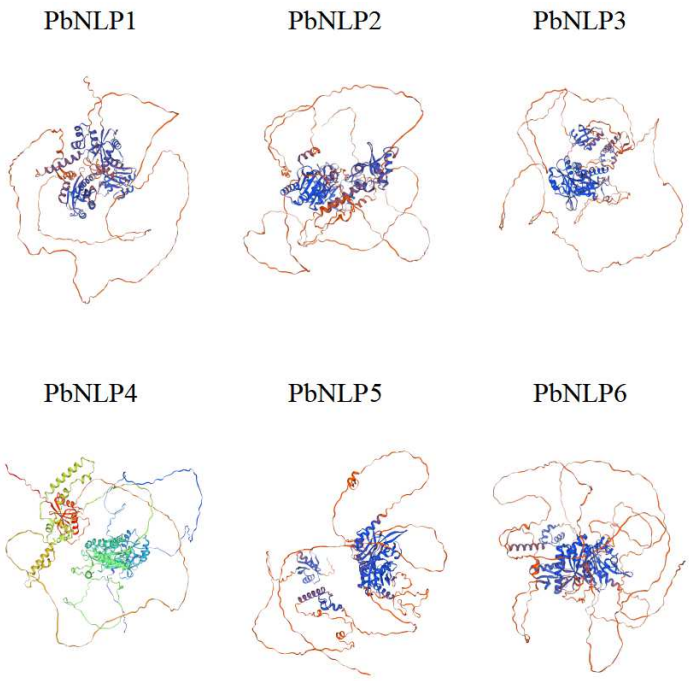


Fig. 7 Tertiary structure models of six representative PbNLP proteins. The blue regions indicate conserved functional domains, and the orange lines represent the remaining polypeptide chains.

GO and KEGG Enrichment Analysis

Our investigation of the biological functions and molecular pathways of the PbNLP gene family was carried out via GO and KEGG enrichment analysis of these sequences (Fig. 8). The GO enrichment finding revealed that the most core functional enrichment of this gene family was all concentrated in the nitrogen metabolism pathway, including nitrate metabolic process, nitrate assimilation and nitrogen cycle metabolic process. Meanwhile, terms associated with transcriptional regulation such as DNA-binding transcription factor activity and transcription regulator activity were also prominently enriched. KEGG enrichment further showed that the gene family were mainly assigned to transcription factors, plant hormone signal transduction and plant signaling pathways. Collectively, these results indicated that the stress triggered extensive transcriptional reprogramming, accompanied by the reprogramming of nitrogen metabolism, and plant hormone-mediated signal transduction might play central roles in the stress response.

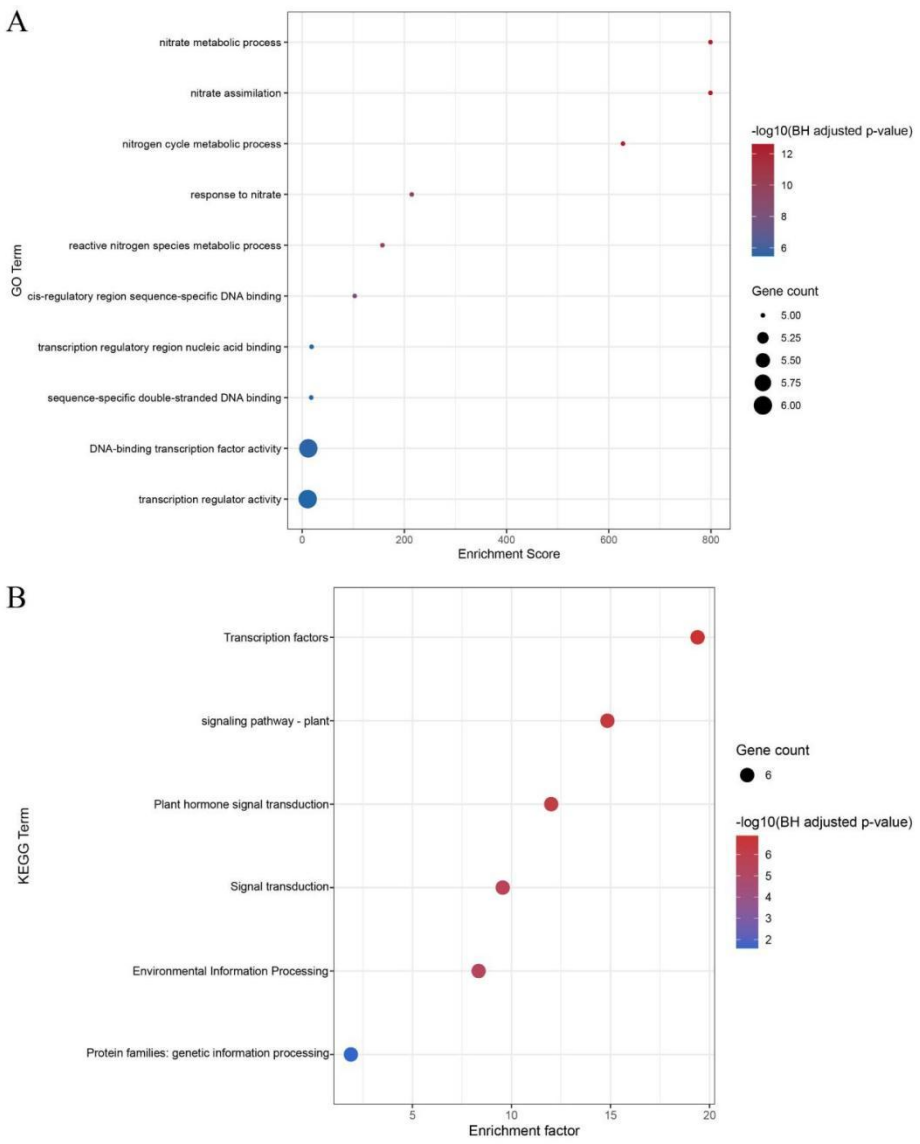


Fig. 8 GO and KEGG enrichment analysis of *PbNLP* gene family. **(a)** GO enrichment bubble plot. X-axis represents enrichment score; colour intensity indicates $-\log_{10}$(BH-adjusted *P*-value); dot size corresponds to gene count. **(b)** KEGG enrichment bubble plot. X-axis represents enrichment factor.

Protein–Protein Interaction Network Prediction of PbNLP Family

In order to investigate the interaction profile of PbNLP family, it might be also feasible to develop a protein-protein interaction network of PbNLP family proteins (Fig. 9). Extensive homologous interactions were detected among PbNLP members, and all PbNLP proteins exhibit strong interactions with the hub protein ROPGEF14. In contrast to the strong interactions with ROPGEF14, the association between PbNLP proteins and RKD1, a conserved reproductive development transcription factor in plants, is notably weaker. Regarding interaction coverage among members, PbNLP3 exhibit the highest connectivity and broadest interaction spectrum within the network, whereas the remaining

members show relatively fewer connections. Furthermore, PbNLP proteins are predicted to broadly interact with ANR1, LBD, TCP, and MOB family proteins, though the interaction affinities vary among different MOB subtypes.

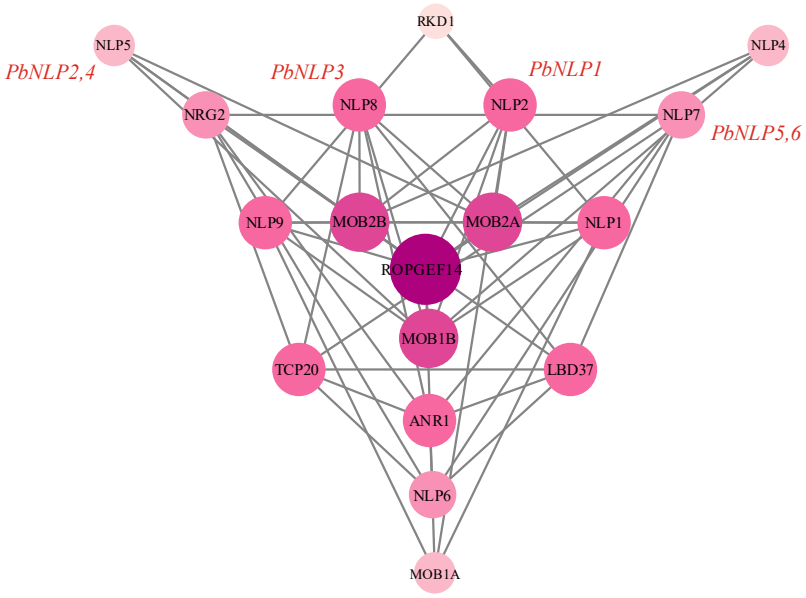


Fig. 9 The interaction network of PbNLP proteins was constructed by using the STRING database and the known homologous proteins of *Arabidopsis thaliana*. The lines connecting two nodes are called edges. Larger circles with darker colors represent a greater number of interacting proteins.

The Tissue-Specific Expression Profile Analysis

This experiment analyzed transcript abundance of 6 *PbNLP* genes across 5 distinct tissues using RNA-seq data of *P. bournei* (Fig. 10). Their results showed a strong tissue-specific expression bias between these genes. On average, leaf and bark expressed significantly more than xylem, especially *PbNLP3* and *PbNLP5*.Similarly, the expression level of *PbNLP1* is relatively low, considering both the level of *PbNLP3* and *PbNLP5*, but it was found that the level of expression in leaves is not similar to the level in other tested tissues. It is important to note that *PbNLP3* was strongly expressed in every tested tissue relative to other genes. Considering the central regulatory role of the *NLP* family in the nitrate signaling pathway, the generalized high expression of *PbNLP3* proposes that it can be considered as an essential regulatory hub of the *P. bournei* nitrogen metabolism network. Nonetheless, the expression of *PbNLP2* and *PbNLP4* in all the tested tissues was low and they might only be activated under certain conditions.

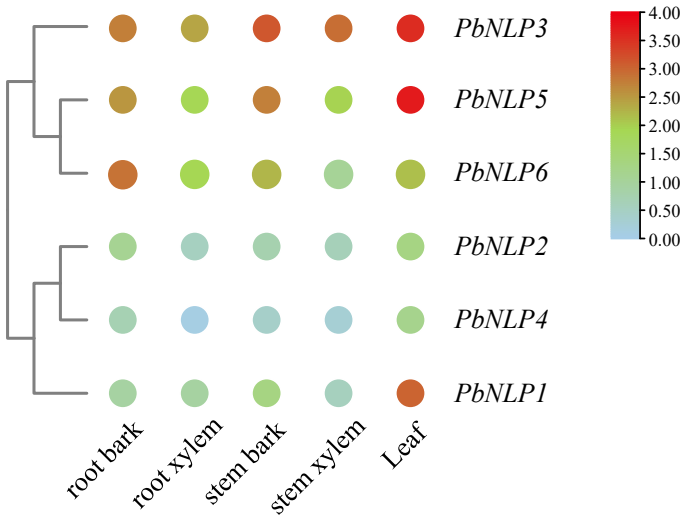


Fig. 10 Tissue-specific expression profiles of *PbNLP* genes across different organs of *Phoebe bournei*. Heatmap visualization shows normalized transcript abundance of each *PbNLP* member in five tested tissues.

The Gene Expression and Correlation Heatmap of *PbNLP* Gene Family

To deeply explore the internal division of labor and synergy within the *PbNLP* gene family while screening for candidate genes for functional validation, we utilized the transcriptional data under heat stress of *P. bournei*, conducting a gene expression and correlation heatmap analysis (Fig. 11). These results showed various patterns of expression for 6 *PbNLP* genes following heat exposure. *PbNLP3* exhibited a high basic expression quantities in the control group, and its expression was further up-regulated after stress treatment, peaking at 12-24 h, indicating that this gene actively responds to stress signals and acts as a key candidate gene for stress response. *PbNLP1* and *PbNLP2* were highly expressed under control conditions, while their transcripts decreased rapidly at 1 h, then sustained in low levels thereafter, suggesting that their transcription was significantly inhibited by stress. *PbNLP5* and *PbNLP6* maintained moderate expression in the early stage of stress and then were gradually down-regulated with prolonged treatment. *PbNLP4* kept extremely low transcript abundance throughout the whole treatment period and was barely induced by stress. In addition, we discovered that the levels of *PbNLP3* and other *PbNLP* genes have negative correlations, which might imply that they have functional differences and could be antagonistic. Excluding *PbNLP3*, other *PbNLP* paralogs exhibited positive pairwise expression correlations that differed in strength. Especially, *PbNLP1* and *PbNLP2*, and *PbNLP1* and *PbNLP5* were strongly correlated. This can be inferred: they are probably involved in common functional pathways and are subject to common transcriptional regulation. In summary, members of the *PbNLP* gene family have undergone expression divergence in stress

responses, with different genes playing distinct roles as positive responders, negatively-regulated factors and non-responsive genes, having different connection between each other.

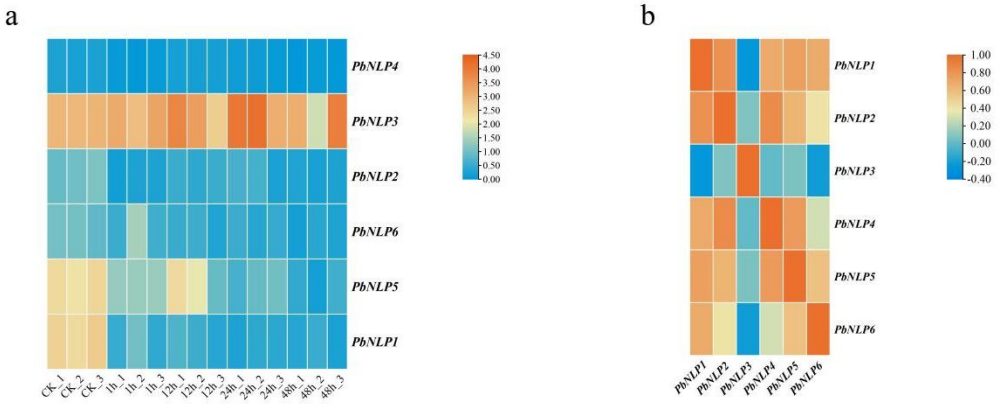


Fig. 11 Gene expression and correlation heatmap analysis of *PbNLP* gene family heat treatment. **(a)** Gene expression heatmap under heat stress during different periods. **(b)** Gene correlation heatmap under heat treatment.

Temporal Expression Dynamics in Response to Heat Stress

To dig out the molecular response of *PbNLP* genes to heat stress while further selecting the key members of the *PbNLP* family, the qRT-PCR technology has been applied to quantify expression changes of five *PbNLP* genes at six intervals post-treatment, namely 0, 1, 3, 6, 12 ,and 24 hours post heat treatment (Fig. 12). Overall expression trends were highly comparable to those of the transcriptome data and this proved the validity of the RNA-seq data. *PbNLP1* expression was reduced to an extreme point after 1 hour of the stress and remained low after 3 hours. After that, the expression increased slightly at 6 hours and then went down at 12 hours. A sharp up-regulation was observed at 24 hours, reaching the maximum value across all time-points and significantly higher than the control. Thus, *PbNLP1* exhibited a typical “early-repressed, late-induced” temporal expression pattern. *PbNLP2* shared highly similar expression dynamics with *PbNLP1*. No significant change was found at 1 hour, whereas its transcription was extremely suppressed from 3 hours to 12 hours. Its expression increased dramatically and peaked at 24 hours with an extremely significant difference compared with CK. *PbNLP3* was gradually induced by stress over time. Its expression was significantly elevated at 1 h, followed by a transient drop to the control level at 3 hours. Continuous up-regulation occurred from 6 hours onwards, reaching the highest level at 24 hours. These findings indicated that *PbNLP3* could actively perceive stress signals, and its induction effect was enhanced with prolonged stress duration. *PbNLP5* displayed an expression pattern analogous to *PbNLP3*. It was significantly and briefly

up-regulated at 1 hour and subsequently returned to baseline at 3 hours. Its transcript abundance was increasing over time between 6-24 hours and peaked at 24 hours, which indicates that it is a stress-inducible gene. *PbNLP4* was strongly suppressed from 1 h to 12 h with the lowest expression at 12 h; although it recovered at 24 h, its induction magnitude was moderate compared with other PbNLP genes. *PbNLP5* showed slight induction at 1 h, subsequent repression at 3 h, gradual accumulation from 6 h to 12 h, and peaked at 24 h. *PbNLP6* showed an overall trend of initial reduction followed by recovery. Its expression was extremely significantly inhibited from 1 h to 12 h, and a distinct rebound occurred at 24 h, though the magnitude of up-regulation was lower than that of the other four genes.

In conclusion, 6 *PbNLP* genes could be classified into two distinct response types under stress. *PbNLP3* and *PbNLP5* belonged to the continuously-up-regulated group, while *PbNLP1*, *PbNLP2*, *PbNLP4* and *PbNLP6* were early-repressed and late-induced genes. These results further suggested expression divergence among *PbNLP* family members, implying that different *PbNLP* paralogs participate in regulatory pathways at early and late phases of stress response, respectively.

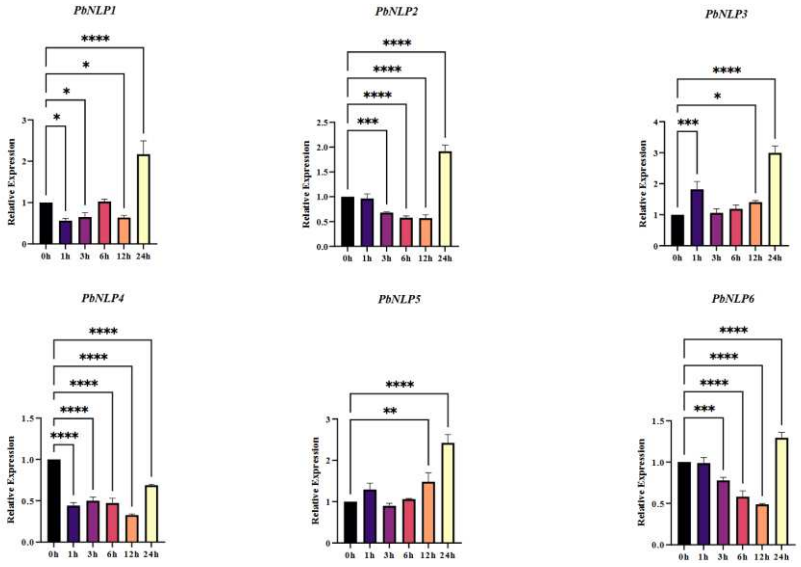


Fig. 12 qRT-PCR expression patterns of *PbNLP* genes in *Phoebe bournei* under heat stress. Relative expression levels of five *PbNLP* genes were detected at 0, 1, 3, 6, 12, and 24 h of heat stress (0 h was set as the basal expression level of 1.0). Error bars represent standard deviations (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Significant differences compared with the 0 h control group.

Heatmap Analysis of Physiological Indicators Under Heat Stress

In order to find out if there is a correlation between *PbNLP* expression and response of leaf photosynthesis-physiology to heat stress, correlation analysis was carried out between 6 *PbNLP*

transcript levels and six photosynthetic parameters, including Chl a (chlorophyll a), Ci (intercellular CO_2 concentration), Gs (stomatal conductance), Fv/Fo (PSII activity), Tr (transpiration rate), and Pn (net photosynthetic rate) (Fig. 13). The heatmap revealed distinct correlation patterns between individual *PbNLP* members and photosynthetic indicators, further suggesting expression divergence within this gene family. *PbNLP1*, *PbNLP5* and *PbNLP6* showed an extremely significant negative association with Ci , while displaying highly significant positive associations with both Fv/Fo and Pn. Such findings implied that greater transcript values of *PbNLP1*, *PbNLP5* and *PbNLP6* were associated with smaller intercellular CO_2 concentration and larger PSII activity and net photosynthetic rate and its expression was highly consistent with photosynthetic capacity. *PbNLP2*, *PbNLP3* and *PbNLP4* showed no significant associations with all examined photosynthetic and physiological parameters, suggesting that their transcriptional changes were not directly linked to the above-mentioned photosynthetic responses under heat stress conditions.

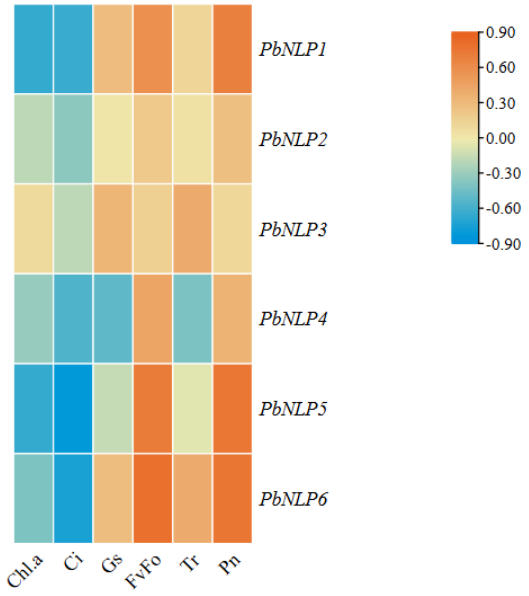


Fig. 13 Correlation heatmap between *PbNLP* genes and photosynthetic physiological indicators in *Phoebe bournei* under heat stress. 6 pivotal photosynthetic related physiological parameters: Chl a, Ci, Gs, Fv/Fo, Tr, and Pn. The color gradient from blue (−0.90) to red (0.90) represents negative to positive correlation coefficients.

Discussion

NLPs are plant specific master transcription factors and regulate the nitrogen signal transduction, yet their unique regulatory modes mediating heat tolerance in woody endangered trees remain insufficiently characterized (Sámano et al. 2024). In this research, we systematically identified 6 *PbNLP* family members and adopted multi-layer bioinformatics combined with qRT-PCR experimental

verification to analyze their physicochemical traits, evolutionary phylogeny, chromosome distribution, gene duplication patterns, gene structure, promoter cis-elements, protein spatial conformation, protein-protein interaction network, GO functional enrichment, tissue-specific transcription, intra-family expression correlation, and dynamic expression changes under continuous heat stress, as well as the correlation between gene abundance and photosynthetic physiological indicators. Comprehensive multi-dimensional data proved that the *PbNLP* family retains highly conserved nitrogen signal regulatory functions and exhibits prominent intra-family subfunctionalization under heat stress, serving as critical molecular nodes for the crosstalk between nitrogen homeostasis and heat adaptation of *P. bournei*.

Evolutionary and Genomic Characteristics of the *PbNLP* Gene Family

In our investigation, 6 *PbNLP* genes were genome-widely isolated and named *PbNLP1* to *PbNLP6*. The number of *NLP* family members in *P. bournei* was moderate when compared with other woody species, such as tea plant (Li et al. 2024). All the transcribed PbNLP proteins are predicted to be localized in the nucleus, where they possess a complete RWP-RK domain for DNA binding and a PB1 domain mediating protein–protein interactions (Cao et al. 2017; Chen et al. 2024; Sámano et al. 2024). Physicochemical calculation revealed obvious property divergence among paralogs: only PbNLP1 was basic protein (pI=8.26), while the other five were acidic. All *PbNLP* genes were unstable hydrophilic proteins, which provides a prerequisite for their flexible transcriptional regulation in the intracellular aqueous environment.

Phylogenetic evaluation of NLP homologues in *P. bournei*, *A. thaliana* and *S. lycopersicum* showed that each *NLP* gene belonged to one of four subgroups. It is also important to note that *PbNLP* genes were classified in Groups A, B, and D except those in Group C. Group D contained three paralogs (*PbNLP1*, *PbNLP2,* and *PbNLP4*), Group B two closely linked genes (*PbNLP5*, *PbNLP6*), and Group A merely a single copy (*PbNLP3*). Every *PbNLP* clustered with dicot orthologs in the same subclade, demonstrating the evolutionary conservation of *NLP* genes across dicotyledonous species. Uneven gene retention among subgroups implied subgroup-specific duplication or gene loss events occurred during the genome evolution of *P. bournei*, which needs further comparative genomic evidence to be validated.

Chromosomal localization results showed 6 *PbNLP* genes scattered on six independent chromosomes without tandem duplication clusters. Intraspecific synteny analysis further clarified that

the main cause of *PbNLP* family expansion was segmental duplication and each could be divided into two syntenic modules and one of them contained an isolate of PbNLP3 where no fragment collinearity was observed. Module A (*PbNLP1*, *PbNLP2,* and *PbNLP4*) may have originated from ancient whole-genome duplication, and Module B (*PbNLP5* and *PbNLP6*) may have derived from independent segmental duplication, whereas *PbNLP3* may have undergone underwent transposon-mediated duplication and massive sequence differentiation, resulting in the disappearance of collinear signals. This polyphyletic expansion pattern generated rich genetic variation reserves for adaptive differentiation under subtropical heat stress.

The interspecific collinearity comparison presented more syntenic gene pairs between two woody plants (*P. bournei* and *P. trichocarpa*, 16 pairs) than for *P. bournei* paired with *A. thaliana* (11 pairs) or rice (9 pairs), which intuitively verified that the *PbNLP* gene family has closer evolutionary affinity with woody dicots, consistent with phylogenetic clustering results.

Gene structure, conserved motifs, and functional domains analysis showed that RWP-RK and PB1 core domains, together with five conserved protein motifs, were highly conserved across all *PbNLP* genes, guaranteeing their basic capacity to sense nitrate signals and interact with partner proteins. Distinct structural divergence mainly existed in untranslated regions (UTRs): *PbNLP5* lacked a UTR entirely, and *PbNLP2* only possessed a 3′ UTR, while *PbNLP1*, *PbNLP3*, *PbNLP4,* and *PbNLP6* owned complete 5′ and 3′ dual UTR sequences. Simplified UTR structure may reduce the complexity of transcriptional regulation and enable rapid gene activation upon stress stimulation, whereas complete UTR sequences support elaborate signal-dependent transcriptional tuning. In addition, *PbNLP5* was positioned near the telomere of Chr07 with high recombination frequency, which may accelerate the variation of its upstream regulatory sequences; nevertheless, the causal link between UTR variation and differential expression patterns still requires subsequent functional verification.

Promoter cis-element prediction screened 19 types of regulatory motifs of each *PbNLP* gene. All genes' promoters contained ABA and anaerobic induction responsive elements; partial members carried gibberellin and MeJA responsive motifs, and *PbNLP4* possessed the maximum number of MYB-binding sites among the whole family. Besides, meristem development-related cis-elements were widely distributed, indicating *PbNLP* genes potentially participate in regulating secondary growth of woody trees (Zhao H et al. 2025). The identification of numerous hormone and stress-responsive cis-elements revealed a strong genomic foundation supporting the dual roles of the *PbNLP* family in

nitrogen signalling and heat stress adaptation.

Protein Conformation, Predicted Biological Functions, and Interaction Network of the *PbNLP* Gene Family

Quantitative statistics of protein secondary structure demonstrated that none of the 6 PbNLPs contained β-turns; random coils occupied the dominant proportion (63.98%–71.75%), followed by α-helices (20.18%–25.36%), and extended strands accounted for the lowest proportion (7.34%–10.66%). The total lack of β-turns and high fraction of random coils suggest an overall flexible conformation for PbNLP proteins, which may favour conformational adaptation when binding ligands or interacting proteins. Small differences in secondary element ratio were observed between members, with PbNLP1 having the largest alpha-helix and extended-strand ratio and PbNLP6 containing the largest random coil percentage, resulting in distinct spatial folding properties. Tertiary structure prediction displayed a unified rigid spherical core and flexible disordered flanks architecture for all PbNLPs. The cores stabilized by α-helices and small extended strands are rigid and cause folding of the PBI and RWP-RK domains to fold for DNA recognition and protein association, whereas extended disordered flanks increase the diversity of interacting partners. The length variation of flexible regions among paralogs matched their secondary structure discrepancy, which explained the differential binding affinities with MOB subtype proteins in the interaction network.

The GO and KEGG enrichment analysis deeply validated core biological functions of the *PbNLP* family. All 6 genes were obviously enriched in nitrogen metabolism-related GO terms, including nitrate metabolic process, nitrate assimilation and nitrogen cycle metabolic process. The answer might be found in the so-called canonical role of NLP transcription factors as previously reported (Krapp et al. 2014; Sámano et al. 2024). Meanwhile, moderate enrichment assigned to transcription factors, plant hormone signal transduction and plant signaling pathways, revealing the *PbNLP* gene family acts as cross-talk hubs connecting nitrogen homeostasis harmone, and heat stress signaling. Such functional feature can easily explain the heterogeneous relationships among various PbNLP transcripts and stomatal conductance, chlorophyll fluorescence, and net photosynthetic rate during heat stress in the future physiological correlation analysis.

PPI network prediction suggested a large amount of homologous interactions between PbNLP members, and with ROPGEF14 emerging as a central hub protein showing strong predixted interactions with all 6 PbNLPs. ROPGEF14 is predicted to regulate ROS burst and subsequent stress

signal cascade during heat stress, suggesting a potential link between PbNLP-regulated nitrogen signaling and heat-evoked ROS response pathways. PbNLP3 exhibited the greatest predicted network connectivity and associated widely with ANR1, LBD and TCP and other MOB family proteins as putative core integrators regulating nitrogen metabolism and other developmental or stress pathways; whereas other PbNLP members appeared to have fewer interaction partners and weaker predicted binding affinity to transcription factor RKD1 involved in reproduction development. In *A. thaliana*, the main intermediates that connect nitrogen and stress signaling are NLP7 and NLP8 (Ding et al. 2022; Huang et al. 2025). *PbNLP3* showed the highest phylogenetic similarity to *AtNLP8*, suggesting that it might play a key role in nitrogen regulation in *P. bournei*.

Tissue Expression Differentiation, Intra-family Synergistic Relationships, and Heat Stress Regulatory and physiological Mechanisms of *PbNLP* gene family

Using transcriptome-based tissue expression profiling, clear tissue-specific patterns of transcription were observed between the members of the *PbNLP* gene family. All the members showed very little transcript abundance in xylems and leaves and bark were the main expression locations. The fact that *PbNLP3* is a widely expressed gene, it can be inferred that it remains highly expressed in all other tissues that were investigated, suggesting that *PbNLP3* functions as an essential regulatory hub in the regulation of nitrogen metabolism homeostasis in *P. bournei*. Whereas *PbNLP2* and *PbNLP4* showed significantly low expression levels in all the tissues tested in comparison to the other genes, they are not constitutively on and may need certain environmental triggers like high-temperature stress to initiate transcription. These tissue-selective expression features are indicative of adaptive properties of this woody species. Bark and leaves are important organs of nitrogen storage and assimilation in trees, and the dominance of *PbNLP*s in these tissues may allow them to respond rapidly to the integration of nitrogen signals with environmental factors. This tissue-specific pattern is consistent with the known seasonal nitrogen remobilization strategy of woody perennials (Gessler et al. 2017), in which bark serves as a major storage site and leaves as the primary assimilation organ, and suggests that *PbNLP* genes may have evolved woody-specific expression features distinct from those of herbaceous model plants.

In heat stress conditions, intra-family correlation analysis also revealed separate synergistic and antagonistic interactions between *PbNLP* paralogs. *PbNLP3* had negative expression correlations with the rest of the family, indicating functional antagonism when exposed to thermal stress. With the

exception of PbNLP3 all other genes were positively correlated, especially *PbNLP1* and *PbNLP2* as well as *PbNLP1* and *PbNLP5* which had very strong positive correlations indicating that these co-regulated genes could be involved in common downstream regulatory cascades when confronted by high temperatures. These coordinated-antagonistic transcriptional relationships highlight the fact that gene-family subfunctionalization is a versatile regulatory arsenal available to woody plants to adapt to heat stress, with various members either working together or canceling out one another to adjust downstream biological responses.

The qRT-PCR validation also supported the validity of the primary transcriptome expression tendencies and separated heat-responsive *PbNLP* genes into two classes of response. *PbNLP3* and *PbNLP5* were part of the continuously up-regulated group, which sensed heat cues in earlier phases and kept high transcripts during stress. *PbNLP1*, *PbNLP2*, *PbNLP4* and *PbNLP6* were described as early repressed but late strongly induced. The early-stage transcriptional downregulation could be seen as an energy-sparing adaptation of *P. bournei*: the first-response genes are activated to activate rapid defence, whereas other paralogs are repressed temporarily to redirect cell resources. Latently induced genes are activated due to the accumulation of heat damage with extended treatment to help repair damages and restore cellular homeostasis. In this staged-response mode, it suggests time-based division of labor between *PbNLP* members under acute heat shock (24 h).

Some partial inconsistencies could be observed in transcriptomic-derived expression heatmap and qRT-PCR quantification results of some *PbNLP* genes. Transcriptome heatmap indicated that *PbNLP5* and *PbNLP6* slowly declined during heat treatment but qRT-PCR analysis indicated that *PbNLP5* and *PbNLP6* strongly increased at the end of late-stress treatment (24h). There are a number of technical and biological reasons that might explain those discrepancies. Firstly, RNA-seq and qRT-PCR experiments were done using different sets of biological samples; the subtle changes in physiology of seedling individuals can change gene transcription patterns during heat stress. Secondly, RNA-seq uses FPKM normalization whereas qRT-PCR uses internal reference gene calibration and various normalization methods can introduce relative expression bias, particularly when working with genes of intermediate or low transcript abundance. Thirdly, the time resolution of sampling and small variations in the level of heat treatment between the two experiment batches could change the point of gene induction or repression. Although these partial discrepancies existed, the main overall tendencies remained largely consistent between the two datasets, which partially corroborates the validity of our

expression measurements. The directionality discrepancy for *PbNLP5/6* may reflect differences in sample batches and normalization methods, and the early-induced classification of *PbNLP5* should therefore be interpreted with caution. This underscores the importance of validating transcriptomic data through qRT-PCR in the study of gene families.

Another correlation analysis performed by physiological pathways in each *PbNLP* member in relation to gene expression and photosynthetic physiological indicators. *PbNLP1*, *PbNLP5* and *PbNLP6* had strong positive correlations on both Pn and Fv/Fo and strong negative correlation on Ci. Together with the fact that it can be induced by heat at an early stage, we conclude that *PbNLP5* may serve as an important integrator connecting nitrogen signaling and photosystem protection: when *PbNLP5* levels are regulated during heat, it enhances the consumption of intercellular CO_2 by carbon fixation and alleviates photoinhibition caused by heat. *PbNLP6* also had strong positive correlations with Pn and Fv/Fo suggesting that it has some degree of functional redundancy with *PbNLP5* in the regulation of photosynthetic performance despite having lower correlation strength. Interestingly, even though *PbNLP3* was highly induced by heat stress, it did not show a significant relationship with any of the measured photosynthetic parameters, indicating that its regulatory process works through physiological pathways unrelated to the photosystem modification, which is compatible with the GO enrichment findings associating it with stomatal movement and water deficit responses. Equally, *PbNLP1*, *PbNLP2* and *PbNLP4* failed to have discernible correlations with the measured photosynthetic indexes. Altogether, these results suggest that only some of the *PbNLP* paralogs directly affect the photosynthetic apparatus in response to heat stress, whereas other ones perform thermotolerance functions through different regulatory modules including nitrogen redistribution and stress signal transduction.

By combining the evolutionary properties, protein-interaction prediction and GO and KEGG enrichment along with multi-omics expression information, a tentative regulatory model of the *PbNLP* mediated nitrogen heat crosstalk molecular physiological process in *P. bournei* could be drawn. Heat-sensitive cis-elements in *PbNLP* promoters sense heat stimuli when exposed to heat cues. Heat-induced ROS signalling associates the PbNLP regulatory module with the hub protein ROPGEF14. Two member genes of the core gene, *PbNLP3* and *PbNLP5*, play different, yet complementary, regulatory functions in order to synchronize heat adaptation. *PbNLP3*, a potemntial key regulator of nitrogen signaling that is highly expressed in many tissues, reacts quickly to heat stress

and helps amplify the stress signals, regulate stomata, and restore the whole plant nitrogen homeostasis. It suppresses most other members of *PbNLP* and controls overall nitrogen redistribution without protecting photosystems. On the other hand, *PbNLP5* is a heat-responsive effector gene, which is very quickly activated at an initial stage of heat stress and becomes the key link between nitrate signaling and photosystem stability. *LlNLP7*, is closely related to *AtNLP7* and positively regulates heat-tolerance in lily. Given that *NLP7* homologs have been implicated in thermotolerance across Arabidopsis, rice, and lily, it is plausible that *PbNLP5* may participate in a similar regulatory module, although this hypothesis requires experimental verification. Previous work has confirmed that *AtNLP7* serves as an important integrator of nitrate signalling and thermotolerance in *Arabidopsis* (Zhao S et al. 2025; Liao et al. 2026). By safeguarding PSII function, *PbNLP5* alleviates heat-derived photodamage and sustains carbon assimilation capacity. Other genes are suppressed in early heat stress and activated at the late stage, functioning as auxiliary regulators for cellular damage repair. In this regulatory cascade, intra-family synergistic and antagonistic interactions fine-tune the amplitude and timing of downstream responses, potentially enabling *P. bournei* to balance nitrogen metabolism, growth maintenance and acute heat stress defence (Fig. 14).

In summary, *PbNLP3* and *PbNLP5* are the candidates genes identified in this study for a potential crosstalk between nitrogen signaling and heat stress responses. According to the previous study, we also speculate that PbNLP proteins may interact with HSF transcription factors to mediate heat stress responses in *P. bournei,* which is valuable for us to have a deep research in the future (Zhu et al. 2025). Nevertheless, all above conclusions are derived from bioinformatic prediction and transcriptional evidence. The exact protein interaction relationships, downstream target genes, and in-vivo physiological functions of *PbNLP3* and *PbNLP5* still require further transgenic overexpression or gene-knockout experiments for functional investigation and verification.

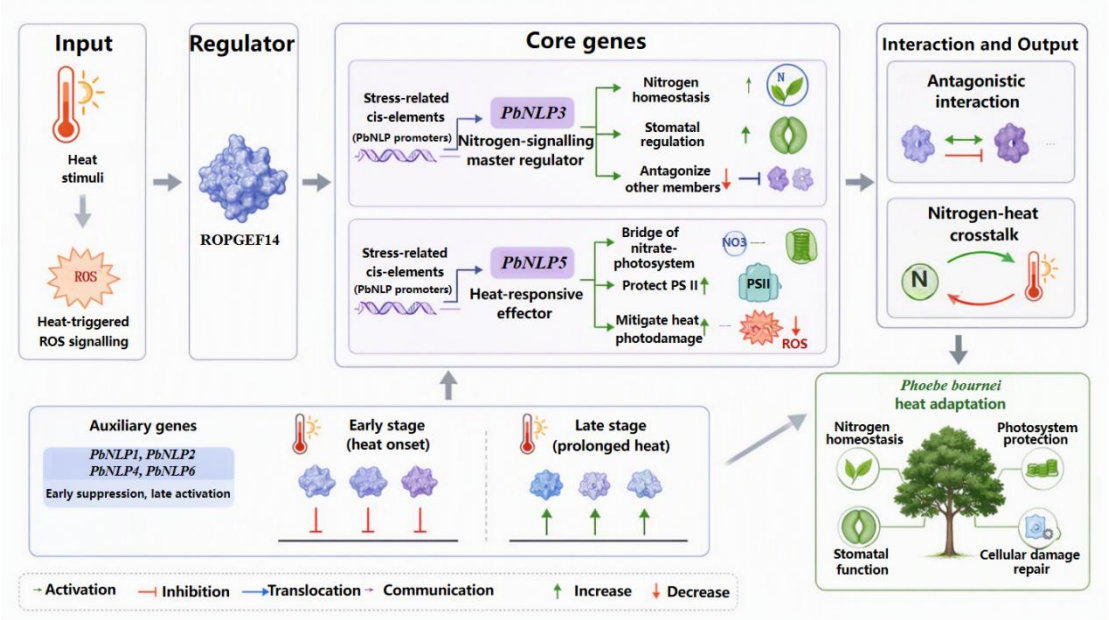


Fig. 14 Schematic regulatory model illustrating the *PbNLP* dependent nitrogen heat signalling module underlying heat stress adaptation in *Phoebe bournei*. Heat-induced ROS signals are transduced via ROPGEF14 to activate *PbNLP* genes. *PbNLP3* regulates nitrogen homeostasis and stomatal responses and antagonizes other family genes, whereas *PbNLP5* protects photosystem II against heat-caused photodamage. *PbNLP1*, *PbNLP2*, *PbNLP4*, *PbNLP6* exhibit early-suppressed and late-activated expression patterns for damage repair. The crosstalk of these pathways facilitates thermotolerance in *Phoebe bournei*.

Conclusion

We have determined and analyzed 6 *PbNLP* genes in *P. bournei*, their phylogenetic relationships, gene architectures, prediction of protein structure and interaction networks, expression profiles and response to heat treatment. Segmental or large-scale duplication contributed to family expansion, and all members retained the conserved RWP-RK and PB1 domains while differing in annotated UTR structure and stress-related promoter cis-elements. Predicted *PbNLP* structures shared a compact core with flexible flanking regions, and interaction network analysis identified ROPGEF14 as a prominent candidate partner. Gene Ontology terms were enriched for nitrogen metabolism, stomatal movement, and responses to water availability. Heat stress *PbNLP* genes were expressed specifically in leaves and bark and have different co-expression and time-dependent responses to heat stress. *PbNLP3* may acted as a a potential key regulator of nitrogen signal across multiple tissues, which rapidly responded to heat stimuli and orchestrates global nitrogen redistribution in plants. *PbNLP5* was induced at the early stage of heat stress and was tightly associated with photosynthetic physiological factors making it a candidate heat-responsive effector gene. These results provide a framework for investigating NLP

mediated links between nitrogen signaling and thermotolerance in woody plants and identify potential targets for heat-tolerance breeding in *P. bournei*.

Supplementary Information The transcriptome data of *P.bournei* under heat stress can be utilized in the *Phoebe bournei* Genome Database (https://pbgenome.org.cn/)

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Authors' Contributions K.Z. integrated the opinions of other authors, finished methodology execution, data collection and analysis, and participated in the manuscript writing revision; C.W. visualized the figures, and participated in the manuscript writing and revision; D.Y. completed data analysis, and participated in the manuscript writing and revision; X.C., S.L.,Y.W. processed manuscript revision and detail checking; Y.Z. and S.C. revised the manuscript and acted as the corresponding authors. They conceived the study, designed the experiment, coordinated all co-authors, and supervised the whole project.

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Data Availability The genomic data utilized in this study to identify the PbNLP gene family is available in public (Accession number: CNP0002030). The transcriptome data used to do the tissue-specific analysis is also available in public (Accession number: PRJNA628065). The transcriptome data of P. bournei under heat stress can be utilized in the Phoebe bournei Genome Database (https://pbgenome.org.cn/), while the database is available. The physiology data used to draw the heatmap of physiological indicators will be available on request to the corresponding author.

Declarations

Conflict of interest There is no conflict of interest need to be declared.

Data Archiving Statement All raw and derived sequence-related datasets generated or analysed in this study have been properly deposited or referenced in publicly accessible repositories following the FAIR (Findable, Accessible, Interoperable, Reusable) data principles, aiming to support full reproducibility of our genome-wide identification, evolutionary characterization and heat-responsive functional analysis of the *PbNLP* gene family in the endangered woody tree *Phoebe bournei*. The complete whole-genome assembly and official gene annotation files of *P. bournei*, which served as core resources for gene family screening, chromosomal localization, intraspecific and interspecific synteny comparisons, as well as gene-structure characterization, are openly available at the China National GeneBank Database under accession number CNP0002030. Transcriptome datasets used for tissue-specific expression profiling across bark, xylem and leaf tissues were retrieved from NCBI BioProject with accession

PRJNA628065. Processed transcriptomic expression matrices under progressive heat-stress treatment supporting differential expression and correlation analysis can be accessed via the *Phoebe bournei* Genome Database (https://pbgenome.org.cn/). Reference protein sequences downloaded from public resources including TAIR, Pfam, NCBI-CDD and Solanaceae Genomics Network were adopted for domain validation and phylogenetic reconstruction without manual modification. Processed qRT-PCR quantification datasets and photosynthetic physiological measurement matrices used for correlation heatmap visualization have not been uploaded to public repositories; these experimental raw datasets can be obtained upon reasonable written request addressed to the corresponding author. No newly generated raw sequencing reads were submitted for this manuscript. Comprehensive archiving information ensures transparent traceability for subsequent bioinformatic re-analysis and experimental replication of our findings concerning nitrogen signalling and heat-response regulatory roles of *PbNLP* genes in this valuable subtropical tree species.

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