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Identification and Transcriptional Regulation of *CAMTA* Genes in *Liriodendron chinense*

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ABSTRACT

This study explores *CAMTA* genes in the rare and endangered Chinese plant species, *Liriodendron chinense*. Despite the completion of whole-genome sequencing, the roles of *CAMTA* genes in calcium regulation and stress responses in this species remain largely unexplored. Within the *L. chinense* genome, we identified two *CAMTA* genes, *Lchi09764* and *Lchi222536*, characterized by four functional domains: CG-1, TIG, ANK repeats, and IQ motifs. Our analyses, including phylogenetic investigations, cis-regulatory element analyses, and chromosomal location studies, aim to elucidate the defining features of *CAMTA* genes in *L. chinense*. Applying Weighted Gene Co-Expression Network Analysis (WGCNA), we explored the impact of *CAMTA* genes on different organs and their regulation under abiotic stress conditions. The identification of significant gene modules and the prediction of promoter binding sites revealed co-expressed genes associated with *CAMTA* transcription factors. In summary, this study provides initial insights into *CAMTA* genes in *L. chinense*, laying the groundwork for future research on their evolution and biological roles. This knowledge enhancement contributes to a better understanding of plant responses to environmental stress—an essential aspect of plant biology.

KEYWORDS

Liriodendron chinense; *CAMTA* genes; abiotic stress; WGCNA; different organs

1 Introduction

In plants, calcium ions (Ca^{2+}) are essential minerals that form an integral part of the cell wall and act as secondary messengers within the cells [1]. Ca^{2+} is essential for controlling a number of physiological functions, such as detecting outside stimuli and preserving the membrane potential across cell membranes [2]. A collection of Ca^{2+}-dependent sensors and modulators are necessary for the Ca^{2+}-mediated signal transduction. Calmodulins (CaMs), calcineurin B-like proteins (CBLs), calmodulin-like proteins (CMLs), calcium-dependent protein kinases (CDPKs), and calcineurin-binding transcription activators (CAMTAs) are some examples of these sensors [3,4].

CAMTAs are transcription factors that are widely distributed in multicellular organisms and have the ability to bind to CaM. Numerous anchor protein repeat sequences that facilitate protein-protein interactions, a Ca^{2+}-dependent CaM-binding domain (CaMBD), a TIG domain involved in non-specific

DNA binding, a CG-1 DNA-binding domain at the N-terminus, and varying numbers of non-Ca-dependent CaM-binding motifs are some of the distinctive structural domains that define them [5]. The roles of the *CAMTA* genes are varied. They have the ability to precisely identify and bind to DNA cis-elements found in downstream genes' promoter regions, controlling the expression of those genes [6]. For instance, EDS1 (Enhanced Disease Susceptibility 1) serves as an immune regulatory factor upstream in the *CAMTA* signaling pathway. In *Arabidopsis thaliana*, CAMTA1 can specifically bind to the CG box in the promoter region of the *EDS1* gene, thereby suppressing its expression [7]. Moreover, *A. thaliana*'s CAMTA3 can bind to the CM2 sequence (CCGCGT) in the *CBF2* gene's promoter region, activating *CBF2* as well as other genes [8]. Moreover, *CAMTAs* are involved in maintaining the pH within vacuoles [9], regulating auxin transport [10], regulating cell division and growth, reacting to outside signal inputs, and taking part in disease resistance [11], modulating fruit development [12] and maturation, and adapting to environmental stressors [13].

For instance, in *A. thaliana*, *AtCAMTA1* and *AtCAMTA5* are expressed during pollen development [14]. Wheat's *CAMTAs* play a role in signal transduction against rust pathogen invasion [15]. In *Brassica campestris*, *BcCAMTAs* exhibit significantly increased expression during cold stress but are suppressed during drought, heavy metal, and nitrate stress [16]. In *Populus trichocarpa*, under short-term mannitol stress, a response is characterized by the upregulation of all 7 *PtCAMTA* genes in both roots and leaves. However, during prolonged treatment, the responses of *PtCAMTA3*, *PtCAMTA6*, and *PtCAMTA7* are relatively subdued [17].

Typically found in the southern Yangtze River region of China, *L. chinense* is a widely dispersed, valuable timber and decorative tree species. *L. chinense*'s genome sequencing offers the chance to identify the *CAMTA* genes, perform bioinformatics analysis, and investigate the transcriptional regulation of these genes. With the help of this work, the biological roles of the *CAMTA* genes in *L. chinense* will hopefully be better understood.

2 Materials and Methods

2.1 The Identification of CAMTA Genes in L. chinense

The genomic and chromosome annotation data for *L. chinense* were obtained from the genome database through downloading (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_003013855.2/).

The *CAMTA* gene and protein sequences from the *A. thaliana* genome database were downloaded from https://www.arabidopsis.org. The *CAMTA* gene and protein sequences from the *Oryza sativa* genome database were obtained from http://rapdb.dna.affrc.go.jp.

The genome sequences from *L. chinense* genome database were downloaded from ftp://ftp.cngb.org. Hidden Markov Model (HMM) files were acquired from the Pfam database (http://pfam.xfam.org/). The TIG domain (PF01833), ANK repeat (PF00023), and IQ motif (PF00612) in the Pfam database were the sources of Hidden Markov Model (HMM) files, which were selected using the E-value $<10^{-5}$ criterion. Protein sequences of *CAMTA* genes from *A. thaliana* and *O. sativa* were utilized in the construction of a Hidden Markov Model.The software for protein domain alignment, HMMER 3.0 (http://hmmer.janelia.org/) was utilized to identify all potential *CAMTA* genes members in the *L. chinense* genome database. We constructed a *CAMTA* genes database for *L. chinense* using the gene sequences from *O. sativa* and *A. thaliana*. The sequences were aligned using the BLAST 2.9.0 + tool, and alignment results were obtained. We'll use the conventional E-value cutoff of 10. These BLAST results were then cross-referenced with the pfam database to acquire a more comprehensive set of data. The last phase resulted in the definitive dataset for *L. chinense*'s *CAMTA* genes. We discovered where the results of the pfam alignment and the BLAST alignment intersected.

2.2 Physicochemical Property Analysis and Phylogenetic Tree Analysis

Basic information about the *L. chinense CAMTA* genes, including protein molecular weight, isoelectric point, and tertiary structure, was analyzed using the ExPASy database (http://www.expasy.org/). Conservation motifs within the *L. chinense CAMTA* genes were identified using the MEME online tool (https://meme-suite.org/meme/tools/meme), and motif diagrams were generated. *A. comosus*, *Carica papaya*, *Solanum lycopersicum*, *Citrus sinensis*, *Theobroma cacao*, *Nicotiana sylvestris*, *P. trichocarpa*, *Brassica napus*, *Malus domestica*, *Nicotiana tomentosiformis*, *Dorcoceras hygrometricum*, *A. thaliana*, *Chlorella variabilis*, *O. sativa CAMTA* sequences were downloaded from the website http://planttfdb.gao-lab.org/. Constructing the phylogenetic tree using the maximum likelihood method was performed with MEGA11 software.

Using the Tbtools toolkit, gene architecture, conserved domains, conserved protein motifs, and chromosomal positions were visualized and examined. Predict cis-acting elements using the PlantCARE software (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/), visualization processing through TBtools version 0.674.

2.3 Transcriptome Data Analysis and qPCR Validation

After being obtained from NCBI, raw transcriptome data (PRJNA559687 [18], PRJNA679089, PRJNA679101 [19]) were obtained from NCBI and underwent quality control using fastp. To do differential gene analysis, the R (version 4.2.2) package Desq2 was used. A padj value less than 0.01 and an absolute |log2foldchange| more than two were used to identify differentially expressed genes for additional research.Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment were predicted using Eggnog-Mapper (http://eggnog-mapper.embl.de/). The GO enrichment analysis database was sourced from http://purl.obolibrary.org/obo/go/go-basic.obo, while the KEGG enrichment analysis database was obtained from https://www.yuque.com/cjchen/hirv8i/ghaxyl. Visualization of the analysis results was conducted using TBtools. Upstream 2000 bp sequences were selected as the promoter and transcription factor binding sites were predicted within the promoter region using https://jaspar.genereg.net/ [20].

The comprehensive repertoire of transcription factors for *A. thaliana* was obtained from http://planttfdb.gao-lab.org/ and utilized for comparative analysis with the complete transcription factor genome of *L. chinense*. Genes demonstrating a Pearson correlation coefficient exceeding 0.6 and a *p*-value below 0.05 were selected as those co-expressed with the *CAMTA* genes.

Furthermore, R version 4.2.2 was used to create a heatmap based on Weighted Gene Co-expression Network Analysis (WGCNA), and Cytoscape v3.10.1 was used to show the co-expression network. Venn diagrams were created via the web-based tool available at http://www.interactivenn.net/. The font colors of the generated figures were manually adjusted using Adobe Illustrator version 28.0. Finally, a heatmap depicting the expression levels of the *CAMTA* gene in *L. chinense* was created using R version 4.2.2.

L. chinense seedlings underwent low-temperature treatment for 0, 1, 3, 6, 12, 24, and 72 h. To ensure the accuracy and reliability of the results, quantitative RT-PCR (qRT-PCR) validation was performed. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression levels of the *CAMTA* genes. The internal reference gene selected for normalization was the 18S gene of *L. chinense*. All qRT-PCR primers were designed using Primer 5.0. The primer design details are provided in Supplementary Table 1.

3 Results

3.1 Identification of the CAMTA Genes in L. chinense

Two *CAMTA* genes have been successfully identified in the genome of *L. chinense*. These genes possess distinct structural domains and exhibit specific biochemical properties, including mild acidity, variability in

stability, and hydrophilicity. Designated as *Lchi09764* and *Lchi22536*, these genes possess four essential functional domains: the CG-1 domain, TIG domain, ANK repeat structure, and IQ motif. They are located on chromosomes Chr4 and Chr3, respectively (Supplementary Tables 2, 3). In the *L. chinense CAMTA* gene, a total of 5 motifs were identified and designated as motif 1, motif 2, motif 3, motif 4, and motif 5 (Supplementary Fig. 1).

3.2 Evolutionary Tree Analysis

An evolutionary tree was constructed using a set of plant species, including *L. chinense* and *A. comosus*, *C. papaya*, *S. lycopersicum*, *C. sinensis*, *T. cacao*, *N. sylvestris*, *P. trichocarpa*, *B. napus*, *M. domestica*, *N. tomentosiformis*, *D. hygrometricum*, *A. thaliana*, *C. variabilis*, *O. sativa* (Fig. 1). Evolutionary tree analysis reveals that the *CAMTA* genes in *L. chinense* are relatively closely related to *O. sativa* and *C. papaya*.

Figure 1: Analysis of the evolutionary tree of *L. chinense CAMTA* genes. Different font colors represent different species

3.3 Identification of the CAMTA Genes in L. chinense

L. chinense CAMTA transcription factor can modulate gene expression by binding to its cis-element during stress response (Supplementary Fig. 2, Supplementary Table 4). The promoter region, which is a sequence approximately 2000 bp upstream of the translation start site, was submitted to PlantCARE to identify cis-elements.

3.4 Transcriptome Analysis

3.4.1 Differential Gene Analysis under Abiotic Stress

According to transcriptome study, there are three main types of stress that cause the expression of *CAMTA* genes: heat, drought, and cold. Nonetheless, it was noted that these genes' expression profiles differed depending on the type of stress. Create a Venn diagram that shows the number of genes that are differentially expressed in response to various abiotic stimuli, as shown in Supplementary Fig. 3. The heatmap of genes co-expressed with *L. chinense CAMTA* genes that are differently expressed is displayed in Supplementary Fig. 4A. The number of differentially expressed genes in *L. chinense* that are upregulated and downregulated in response to different abiotic stress treatments is displayed in a bar chart in Supplementary Fig. 4B. Based on Pearson correlation coefficient-based co-expression network analysis, it can be inferred that the *L. chinense CAMTA* genes may co-regulate abiotic stress responses alongside transcription factors such as BES1, C3H, ERF, GATA, LSD, MYB-related, NAC, SBP, WRKY, and YABBY (Fig. 2).
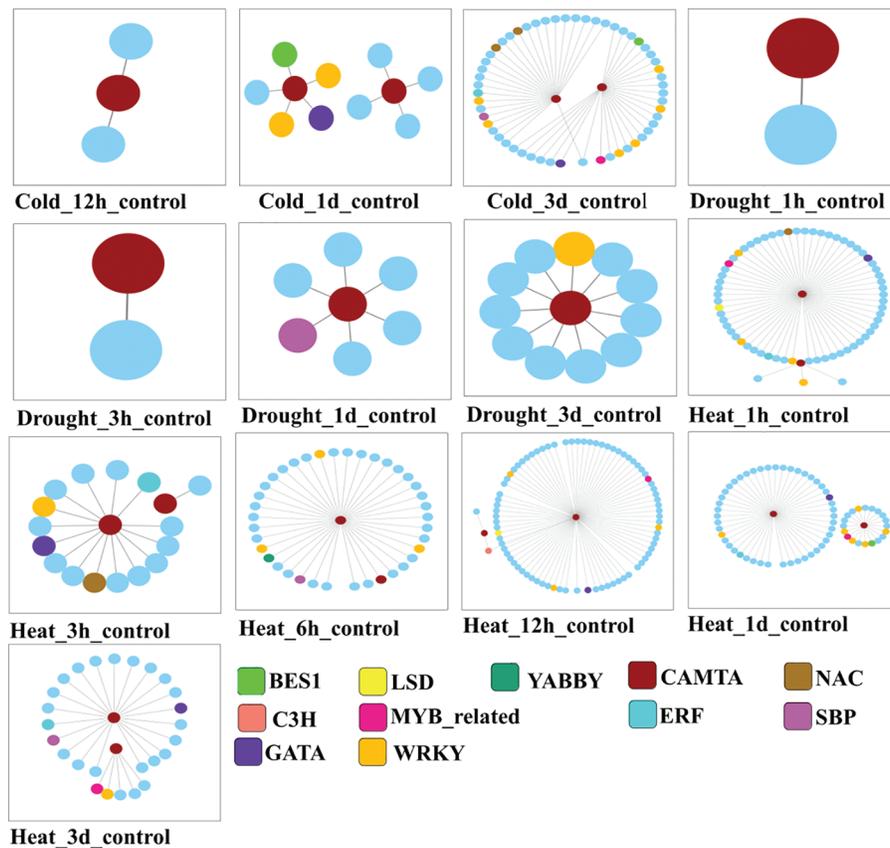
Figure 2: *L. chinense CAMTA* genes and co-expression dynamics under abiotic stress. Under each abiotic stress treatment, the nodes reflect differentially expressed genes of *L. chinense*, and the various colors correspond to different matching transcription factors. Co-expression is indicated by the connecting lines. The association between genes with variable expression is shown by the lines

3.4.2 Enrichment Analysis of Differentially Expressed Genes Co-Expressed with the L. chinense CAMTA Genes

We performed GO and KEGG enrichment analyses on differentially expressed genes responsive to abiotic stress and co-expressed with *L. chinense CAMTA* genes (Fig. 3). These differentially expressed genes that co-expressed the *CAMTA* genes were shown to be enriched in terms of Molecular Function, Cellular Component, and Biological Process, with the highest level of enrichment observed in the GO enrichment analysis. This implies that a number of elements of plant growth and development are regulated by the *L. chinense CAMTA* genes. This gene set's involvement in several biological processes, such as glycosylation, RNA biology, cytoskeleton organization, chromosomal structure and function, and gene expression, was shown by the KEGG enrichment analysis.

### 3.4.3 WGCNA Analysis under Abiotic Stress Conditions

WGCNA was performed on the differentially expressed genes of *L. chinense* under abiotic stress, revealing a total of 4642 genes with altered expression patterns (Supplementary Fig. 5). Originally, only genes with variances larger than the fourth percentile were kept in the gene selection process. We then used the Pearson correlation coefficient to do a sample clustering study and produced a dendrogram that illustrates the sample clustering tree. (Supplementary Fig. 5A). To construct an unscaled network, a soft threshold of 16 was chosen (Supplementary Fig. 5B), leading to the generation of an adjacency matrix and the creation of a topological overlap matrix (Supplementary Fig. 6A). Six unique gene modules were successfully found by WGCNA; these gene modules are distinguished by genes that have similar expression patterns (Supplementary Fig. 6B). This approach greatly enhances our comprehension of the interrelationships and expression profiles of these genes.

Additionally, we chose modules whose sample characteristics had correlation coefficients higher than 0.5. The brown, green, blue, and yellow modules made up these modules. Interestingly, we found differentially expressed genes that co-expressed with *CAMTA* genes within the blue module. Thus, we put up the theory that the blue module's genes and the *CAMTA* genes in *L. chinense* may work together to coordinate the plant's reaction to a 6-h heat stress. Genes co-expressed with *CAMTA* under non-biological stress are found in the blue module. We used Cytoscape to create a network graph (Fig. 4). A diagram displaying the genes found in the blue module. This thorough investigation may reveal important relationships between genes and stress reactions, expanding our knowledge in this area. The prediction of transcription factor binding sites in the upstream promoter regions of genes in the blue module reveals that *Lchi10447* and *Lchi18769* are not only co-expressed with the *L. chinense CAMTA* transcription factor but also have *CAMTA* transcription factor binding sites in their upstream regions (Supplementary Tables 5, 6, Supplementary Fig. 7).

3.4.4 Differential Gene Expression Analysis for Different Organs Using WGCNA

We carried out a WGCNA on a set of 4943 differential genes, obtained from various organs including bract, leaf, petal, pistil, shoot, sepal, and stamen, in order to further investigate the regulatory roles of the *CAMTA* genes in the development of different organs in *L. chinense* (Supplementary Fig. 8).

First, we kept genes whose variances were higher than the fourth quartile criteria in order to undertake gene selection. The Pearson correlation coefficient was then used to perform sample clustering analysis, which led to the creation of a sample clustering tree (Supplementary Fig. 8A). To establish an unscaled network, we selected a soft threshold of 18 (Supplementary Fig. 8B). Following this step, we generated an adjacency matrix and constructed a topological overlap matrix, as illustrated in the (Supplementary Fig. 9A). WGCNA successfully identified six gene modules, each comprising genes with analogous expression patterns, thereby enhancing our comprehension of the correlations and expression profiles among these genes (Supplementary Fig. 9B).
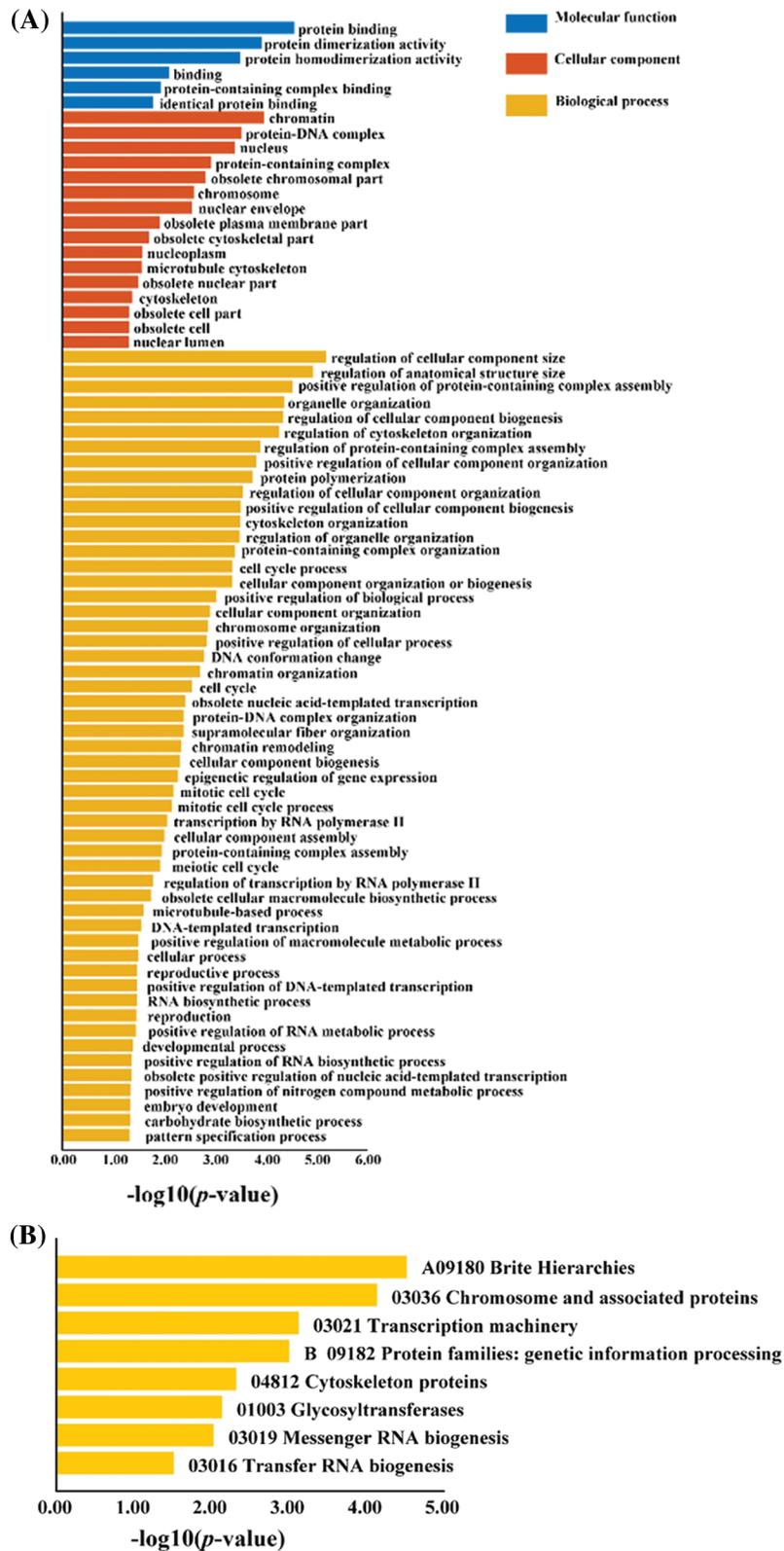
Figure 3: GO enrichment and KEGG enrichment analysis of differentially expressed genes co-expressed with the *L. chinense CAMTA* genes
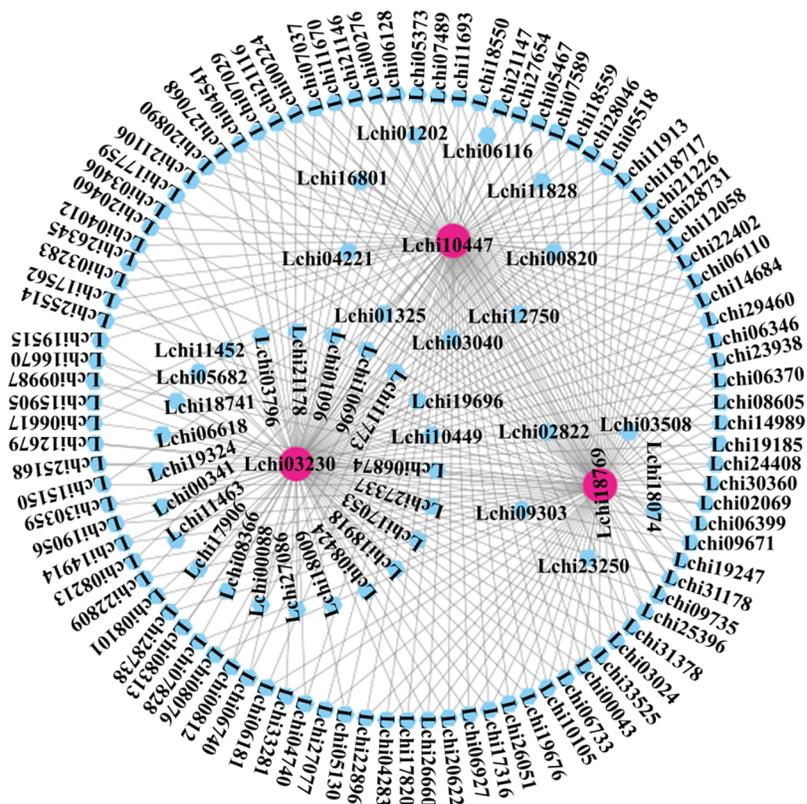
Figure 4: Gene co-expression network plot within the blue module of WGCNA. Differentially expressed genes under abiotic stress conditions are represented by the nodes in the network. Genes that co-express with the *L. chinense CAMTA* genes are shown by pink nodes. The correlation between pairs of differentially expressed genes is shown by the connecting lines

To begin gene selection, we first retained genes whose variances above the fourth quartile threshold. Following sample clustering analysis using the Pearson correlation coefficient, a sample clustering tree was produced. Significantly elevated correlation coefficients (Supplementary Fig. 10). And an analysis of motifs in the upstream promoter sequences was conducted (Supplementary Fig. 11). There are 161 genes altogether in the yellow module. This strategy has great potential to further our knowledge of how the *CAMTA* genes in *L. chinense* regulate relevant genes' transcriptional expression throughout leaf development.

The upstream 2000 bp sequences of genes in the yellow module were extracted for further analysis. Motif analysis was conducted, and the prediction of transcription factor binding sites was performed using JASPAR (Supplementary Tables 7, 8). The presence of *CAMTA* binding sites within the promoter regions of *Lchi14980* and *Lchi01238* has been identified.

3.5 qRT-PCR

To further validate the transcriptional regulation by the *CAMTA* genes in the *L. chinense*, we conducted qRT-PCR validation following different time points of cold stress treatment (1, 3, 6, 12, 24 h, 3 d). The qRT-PCR results revealed a distinct expression pattern for *Lchi09764* and *Lchi22536*. These genes initially exhibited an upregulation in response to cold stress, followed by a subsequent decrease, and then a subsequent increase as the duration of cold stress treatment extended (Supplementary Fig. 12).

4 Discussion

We may now go on to the *CAMTA* genes, which actively contribute to the regulation of these processes, after talking about calcium signals and their function in plants' responses to stressors. In particular, the *CAMTA* gene family members display a range of actions in response to several biotic and abiotic stressors, such as temperature and light [21], salinity [22], hormones [23], and pathogens [24]. This family of transcription factors plays a crucial role in orchestrating plant growth and development under challenging environmental conditions. Researchers have identified this transcription factor in various species, including seven members in *Populus* [17], eight in *Phaseolus vulgaris* [25], fifteen in wheat [26], and ten in *Heimia myrtifolia* [27]. The *CAMTA* genes exhibit slight variations across different species. In our study, we identified two *CAMTA* genes in *L. chinense*, and these identified genes may exhibit distinct functionalities.

Since the *CAMTA* gene is involved in both the control of abiotic stress responses and plant growth and development, it serves a dual role in various plant species. For example, in *A. thaliana*, *AtCAMTA3* expression levels are downregulated during fruit development but significantly upregulated during fruit ripening. *AtCAMTA5* is essential for the formation of pollen [28], whereas *AtCAMTA6* is a key regulator of seed germination [29]. In tea plants, *CsCAMTAs* play a crucial role in regulating the aging process and flowering period [30]. In tomatoes, there is a high differential expression during both fruit development and ripening processes [31]. In *Musa acuminata*, all *MuCAMTA* genes exhibit upregulation under drought stress [32]. Our research highlights the critical function that the *CAMTA* genes perform in *L. chinense*. In addition to components associated with the regulation of diurnal cycles and light responsiveness, this gene also contains cis-acting elements relevant to meristem expression. Notably, under non-biotic stress circumstances, the *CAMTA* genes in *L. chinense* co-express with differently expressed genes, providing more evidence for their potential involvement in regulating plant growth and development. In academic discourse, these discoveries offer significant new information for a fuller understanding of plants' capacity for environmental adaptation. The effects of low temperature stress are substantial in the field of plant biology because they have a major influence on various processes related to growth and development.

Consequently, there is a need to establish intricate regulatory networks that confer resilience and adaptability that enable plants to withstand and adapt to the challenges posed by cold conditions [33]. In *A. thaliana*, the dehydration-responsive element (DRE) and C-repeat motif (C-REPEAT) are crucial elements for the expression of cold-induced genes. The DRE-binding DREB1/CBF transcription factors play a major regulatory role in cold responses, and their absence results in reduced cold tolerance in plants [34]. In *A. thaliana* cold stress, *CAMTA3* and *CAMTA5* induce DREB1B/C via temperature/Ca^{2+}, and RVE4/8 activates DREB1A/C expression during daylight, slightly activating DREB1B regardless of temperature. RVE4/8 uses EE/EE-like sequences, while *CAMTA3* and *CAMTA5* use CGCG-box/CGCG-like sequences [34]. *CAMTA* transcription factors may collaborate with other transcription factors such as NAC, MYB, bZIP, CBF, and WRKY during cold stress [35]. In *A. thaliana*, the mutation of *CAMTA3* leads to a significant reduction in *CBF2* and other cold-induced genes under cold conditions [36]. In tea plants, the expression levels of *CsCAMTA1/3/4/6* are higher in cold-tolerant varieties than in cold-sensitive ones, indicating their crucial role throughout the entire cold acclimation process [30]. In the context of the current research, understanding the regulatory mechanisms of the *CAMTA* genes in cold stress becomes crucial for the *L. chinense*. This study validated the response of the *L. chinense CAMTA* genes to cold stress at different time points through qRT-PCR. For high-temperature and drought conditions, a more in-depth analysis may be required in future studies.

The *CAMTA* genes are crucial for immunological responses because they actively regulate calcium ion levels and stress reactions [37]. In *A. thaliana*, the *CAMTA3* mutant demonstrates constitutive immunity under low-temperature conditions, whereas the functionally acquired *CAMTA3-3D* mutant exhibits a

markedly diminished disease resistance [38]. In tomatoes, upon infection with *Botrytis cinerea*, all *CAMTA* genes manifest an upregulated expression pattern in leaf tissues [39]. Unfortunately, despite our thorough bioinformatics investigation of the *CAMTA* genes in *L. chinense*, no immune-related functions were found. This discovery deviates from the immune-related roles found in other species' *CAMTA* genes. This discrepancy could be caused by variables peculiar to a particular species or by special *CAMTA* gene regulation mechanisms in *L. chinense* immune responses. Further research is necessary to confirm these findings with greater rigor, explore the specific function of the *CAMTA* genes in immune control in *L. chinense*, and develop a more thorough knowledge of their significance in plant biology. It is noteworthy that the *CAMTA* genes is intricately linked with hormone regulation [33]. In *Cucumis sativus*, a majority of *CAMTA* genes possess diverse hormone-responsive elements and stress-related motifs, exhibiting varying expression patterns across different tissues and inducibility by hormones such as IAA and ABA [40]. Similarly, in tea plants, *CAMTA* genes harbor cis-acting elements related to hormone regulation [41]. The promoter regions of *CitCAMTAs* in *Citrus sinensis* contain cis-acting elements responsive to growth hormones, including Auxin Response Elements (AuxR-core) and TGA elements [42]. In *Linum usitatissimum*, the promoter regions of *LuCAMTAs* contain Abscisic Acid Response Elements (ABRE) and Salicylic Acid Response Elements (TCA elements) [43]. In this study, putative cis-acting elements associated with hormone responses within the *L. chinense CAMTA* genes have been predicted.

However, it is crucial to stress that further experimental validation is required for these first investigations. More research is necessary to confirm the precise involvement of the *CAMTA* genes in various biological processes, particularly in the areas of hormone control, stress response, and physiological and biochemical regulatory mechanisms.

5 Conclusions

This study found two *CAMTA* genes in *L. chinense* (*Lchi09764* and *Lchi22536*). Bioinformatics analyses covered gene structure, evolution, organ regulation, and responses to abiotic stress. WGCNA analysis highlighted key co-regulated genes in organ development and abiotic stress, with *Lchi10447* and *Lchi18769* identified as pivotal co-expressed with *CAMTA*. Additionally, WGCNA emphasized the importance of *Lchi14980* and *Lchi01238* in coordinating organ development with *CAMTA*, linked to the yellow module related to leaf growth. These findings deepen our understanding of *L. chinense CAMTA* in regulating abiotic stress responses and organ growth, validated by qPCR under cold stress indicating significant regulatory roles for *CAMTA* genes.

Author Contributions: K.H. conducted a literature review and drafted the manuscript. Y.R. and J.C. examined and edited the manuscript. T.M. provided assistance with image modifications, data analysis, and language polishing. L.Y. secured funding and supervised the progress of manuscript.

Availability of Data and Materials: The datasets analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

Supplementary Materials: The supplementary material is available online at https://doi.org/10.32604/phyton.2024.047739.

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