Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2025.060566

ARTICLE

Genome-Wide Identification and Expression Analysis of Calmodulin-Like Proteins in Tobacco

Mengjie Xu, Anbin Wang, Tonghong Zuo, Hecui Zhang, Zhihao Hu and Liquan Zhu*

College of Agronomy and Biotechnology, Southwest University, Chongqing, 400715, China

*Corresponding Author: Liquan Zhu. Email: zhuliquan@swu.edu.cn

Received: 04 November 2024 Accepted: 05 December 2024 Published: 24 January 2025

ABSTRACT

Calmodulin-like (CMLs) proteins are critical in calcium signaling and essential for plant growth, development, and stress responses. In many species, the CMLs families have been identified and described. However, the characterization and expression profiling of *CMLs* genes in tobacco is retrievable. In this study, a comprehensive whole-genome identification and analysis, and 75 *NtCML* genes were identified in tobacco, each containing two to four EF-hand domains. Most NtCML proteins exhibited conserved gene structures and motifs. Notably, most NtCML proteins were intron-less and distributed across 18 chromosomes. Two pairs of tandemly duplicated genes and seven pairs of segmentally duplicated genes were identified within the tobacco genome. Furthermore, 22 pairs of orthologous *CMLs* genes were discovered between Arabidopsis and tobacco. Cis-acting element analysis revealed that elements associated with hormones, stress responses, and plant growth and development were found in the promoter regions. Expression analysis indicated that some *NtCML* genes displayed tissue-specific expression patterns. Specifically, *NtCML12*, *NtCML18*, *NtCML27*, and *NtCML28* showed significant upregulation during cold acclimation treatment. These results indicate that tobacco CMLs act as Ca^{2+} signal transducers, regulating plant growth and abiotic stress responses.

KEYWORDS

Calmodulin-like (CMLs); tobacco; plant growth; abiotic stress

1 Introduction

Calcium ions (Ca^{2+}) are essential for plants, serving as structural componentd of cell walls and membranes, as well as vital secondary messengers. Plant growth, development, and adaptability to biotic and abiotic stresses are significantly influenced by calcium ions [1]. Different abiotic and biotic stresses, including hormones, temperature, drought, salt, disease, and so on, can change variations in the level of cytoplasmic calcium ions and affect the movements of calcium ions in plant cells [1,2]. Calcium-binding proteins can sense and interpret intracellular calcium concentrations before undergoing conformational changes and interacting with downstream signaling partners, supporting the plant in responding to environmental stresses [3,4]. Calcium-binding proteins act as calcium sensors, and there are four kinds of calcium ion sensors of plants: calmodulins (CaMs), calmodulin-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs), which contain one or more EF-hand motifs [5–8]. They commonly contain elongation factor hand (EF-hand) motifs. The EF-hand,

Copyright © 2025 The Authors. Published by Tech Science Press.
This work is licensed under a Creative Commons Attribution 4.0 International License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

characterized by a helix-loop-helix structure, can bind calcium ions and subsequently undergo a conformational change. This alteration allows it to interact with downstream proteins and adjust its catalytic activity, which includes gene regulation, protein interactions, protein phosphorylation, and metabolic changes [9–11]. Plant CaMs have four EF-hand domains, whereas CMLs have one to six and no extra functional domains. CMLs typically have 16%–75% of their amino acid composition in common with CaMs [12].

The CMLs genes in *Arabidopsis thaliana* and rice currently exhibit 50 and 32 genes, respectively [12,13]. Since the completion of various plant genome sequencing projects, the *CMLs* gene family has been identified in numerous other plant species, including tomato [14], cucumber [10], apple [4], grape [11], chrysanthemum [15], barley [16]. Previous research has shown that the genes of CMLs play a significant role in plant development, growth, and stress responses [17]. *CML39, CML24*, and *CML25* are crucial calcium ion sensors in plant growth and development [18–20]. *CML9, CML8, and CML41* are involved in plant immune response [21–23]. *CML9*, *CML20, CML24*, *CML37*, *CML38*, and *CML39* participate in plant salt stress response [24–27]. *MtCML42* regulates flowering time and cold tolerance by gradually increasing MtFTa expression and decreasing MtABI5 [28]. *SlCML39* is a significant negative regulator for high-temperature tolerance [29]. Overexpression of *ShCML44* demonstrated increased resistance to salt, drought, and cold stress [30]. *CMLs* genes are involved in multiple physiological functions.

Tobacco is a significant cash crop and model organism worldwide. Although several NtCMLs have been reported in various publications, a comprehensive examination of tobacco CMLs has yet to be conducted. In this study, 75 *NtCMLs* genes were identified in the tobacco genome. Comprehensive analyses including gene structure, chromosomal distribution, gene duplications, motifs or domains, cis-acting elements, evolutionary relationships, organ-based gene expression profiles, and under cold acclimation conditions. The findings might provide important insights into physiological and molecular research on the *NtCMLs* genes.

2 Materials and Methods

2.1 Identification of NtCMLs Genes and Sequence Analysis

The published *CMLs* gene sequences in *Arabidopsis* and rice were obtained from the TAIR database (https://www.arabidopsis.org) (accessed on 04 December 2024) and the TIGR database (http://rice.plantbiology.msu.edu) to identify members of the *CMLs* gene family in the tobacco genome. Then, 32 *OsCMLs* and 50 *AtCMLs* proteins were used as query sequences to perform BLASTP search (E-value < e − 5), and the redundant and repetitive sequences were removed manually. Meanwhile, the HMMER (PF13499) was utilized as a keyword in the databases above to conduct searches. The NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd) (accessed on 04 December 2024), InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) (accessed on 04 December 2024), and SMART (http://smart.embl-heidelberg.de/) (accessed on 04 December 2024) were used to predict the structural domains of EF-hands, eliminating protein sequences that lack EF-hands or contain other functional domains. Additionally, *AtCaM2* was used to guarantee the NtCMLs by acting as usual CaMs and ensuring that the amino acid identity was less than 80%. The nucleotide and predicted amino acid sequences of the discovered genes, which were named *NtCML1* through *NtCML75*, were used for additional investigation.

ExPASyProtParam (http://web.expasy.org/protparm/) (accessed on 04 December 2024) was used to estimate the physicochemical properties of NtCMLs, such as the number of amino acids, theoretical point (pI), grand average of hydropathicity (GRAVY), and aliphatic index. SMART was used to forecast the number of EF-hands (https://smart.embl.de/smart/set_mode.cgi?NORMAL=1) (accessed on 04 December 2024). Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) (accessed on 04 December 2024) was used to predict subcellular localization.

2.2 Gene Structure and Conserved Motif Analysis

MEME (http://meme-suite.org/index.html) (accessed on 04 December 2024) was used to study the conserved domains, and ten motifs were chosen. The exon and intron structures of NtCMLs were ascertained using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (accessed on 04 December 2024).

2.3 Phylogenetic Analysis

Using 1000 bootstrap replicates and the neighbor-joining method in MEGA7, the phylogenetic tree was constructed for evolutionary analysis. The NtCML proteins were classified based on the evolutionary relationships of 50 AtCML proteins. Evolutionary tree landscaping can be achieved using the website ChiPlot (https://www.chiplot.online/) (accessed on 04 December 2024).

2.4 Cis-Acting Elements Analysis

PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) (accessed on 04 December 2024) was used to examine the 2000 bp upstream sequences of *NtCMLs* genes to identify the cis-acting elements in the promoter region of *NtCMLs* genes. The Simple BioSequence Viewer from TBtools is used to visualize.

2.5 Chromosome Localisation and Collinearity Analysis

The online website mg2c (http://mg2c.iask.in/mg2c_v2.1/) (accessed on 04 December 2024) was utilized to visualize the chromosomal position. TBtools software was used to examine the homologous relationship of *NtCML* genes. The Multiple Collinearity Scan toolbox (MCScanX) examined gene duplication occurrences.

2.6 Tissue Specifc Expression Analysis

Tobacco K326 tissue data from 19 tissues was downloaded from the EMBL-EBI website. The corresponding gene expression profile data were obtained by comparing the sample numbers of *NtCMLs* gene family members. The data was plotted using the Heat Map tool in the software TBtools.

2.7 Cold Acclimation Stress Treatments

The tobacco cultivar ‘Yunyan87’ was sown in soil and allowed to grow at room temperature (26°C) until the plants had six or seven leaves. The plants were then divided into two groups. The P seedlings were grown in an artificial climate chamber and were treated with cold acclimation therapy for three days at a temperature of 12°C (night/day). The other group of seedlings that were not cold-acclimated is known as N. The N and P seedling groups were treated normally for 7 days and recorded as NCT and PCT. NCT and PCT seedlings were put through cold treatment (8°C) for three days and recorded as NCL and PCL. Each treatment involved the collection of three biological samples, which were immediately frozen in liquid nitrogen and stored at −80°C until they could be examined further.

2.8 RNA Extraction and Gene Expression Analysis Using Real-Time PCR

The Spin Column Plant Total RNA Purification Kit (Sangon Biotech, Shanghai, China) was used after the frozen samples had been thoroughly ground to powder in liquid nitrogen. 1% agarose gel electrophoresis was used to assess the quality of the RNA. The All-In-One 5X RT Master Mix (ABM, Shanghai, China) is then used for the cDNA synthesis. The qPrimerDB qPCR Primer Database online resource was utilized to design the primers used in the RT-qPCR. Table 1 lists the particular primers used for qRT-PCR. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out in real-time

utilizing a CFX-1000 Real-Time System (BioRad) and SYBR Premix Ex Taq II (TaKaRa, China). Three duplicates of the real-time PCR were conducted. We used the N, NCT, and NCL treatments as the baseline to calculate P, PCT, and PCL expression. The N, NCT, and NCL treatments were utilized as a baseline to calculate P, PCT, and PCL expression. The e $2^{-\Delta\Delta Ct}$ technique was used to analyze relative expression [31]. The data shows the average of three biological replicates.

Table 1: Primers used in RT-qPCR reactions

Gene	Forward primer	Reverse primer
Nt26	GAAGAAGGTCCCAAGGGTTC	TCTCCTTTAACACCAACGG
NtCML3	GTTCTGTGCGCTTTGGGATTAA	TCCTGGAAATCGATTCTGCCAT
NtCML8	GCAAGATTTCACCAGGGGAGTA	AAATCCGTCACCATCTGCATCT
NtCML12	GCAAGAATGACAACCTTTGGCT	GTACAACCAAGAGCTTGTCTGC
NtCML18	GACAACAATGGCAACGGATTCA	TTAGCCATTGATCCAGCCAACT
NtCML22	TTTCATTGTCCCGTTGAAAGCC	AGTTAACACTACCGTCACCGTC
NtCML23	ATTCGAGAGATTCCTCACAGCC	TAGCATCATTCGCTTTGCTTCG
NtCML27	TGTGCCTTTGCTAATTCATGGC	ACTTCTTCCCTGCATAGGCTTC
NtCML28	ATGGCGACGGAAAAATCTCTCT	GCCATCAGTGTCAACTTCCAAC
NtCML30	AGAAGAAGAAGAGCTTGCCCAA	CGTTTCCTAGGCTAGCCATGAT
NtCML53	ATTTGCAGGCGAACAGATTACG	CGGCAGAAGAAATGTCACGATC

2.9 Protein Interaction Network Prediction

A network of 75 NtCMLs protein sequences was analyzed using the STRING online server (https://cn.string-db.org/) (accessed on 04 December 2024).

2.10 Statistical Analysis

SPSS26.0 data processing software is utilized to perform variance analysis and significance experiments on experimental data. Origin2021 software is employed to perform mapping analysis on experimental data.

3 Results and Analysis

3.1 Identification and Characterization of NtCMLs Family Members in Tobacco

A total of 75 *NtCMLs* genes were retrieved from the genome of Tobacco and were named in the order of their chromosomal locations (*NtCML1* to *NtCML40* and *NtCML41* to *NtCML75*) (Table 2). The results were confirmed by analyzing the deduced peptides using Pfam, InterProScan, and SMART databases. Physicochemical property analysis showed that the number of amino acids in the NtCMLs proteins ranged from 79 AA (*NtCML39*) to 283 AA (*NtCML6*) and the pI ranged from 3.97 (*NtCML41*) to 9.63 (*NtCML10*). The Aliphatic index ranged from 58.10 (*NtCML29*) to 104.84 (*NtCML57*). The NtCMLs shared 23%~78% identity with *AtCaM2*. Most NtCMLs proteins contained two to four EF-hand domains. The GRAVY values of most NtCMLs proteins were negative, indicating that NtCMLs proteins in cucumber are hydrophilic. Subcellular localization demonstrates that most NtCMLs are found in the cell membrane and cytoplasm.

Table 2: *NtCMLs* genes in the *Nicotiana tabacum* genome and sequence characteristics of the corresponding proteins

Name	Gene ID	EF-hands	AtCaM2 (%)	Number of amino acids	pI	Aliphatic index	GRAVY	Subcellular localization
NtCML1	Nitab4.5_0001622g0090.1	4	26.75	227	4.47	77.31	−0.457	Cell membrane. Cytoplasm. Nucleu.
NtCML2	Nitab4.5_0000356g0070.1	3	32.47	189	4.45	78.94	−0.421	Cell membrane.
NtCML3	Nitab4.5_0002980g0030.1	2	40.91	188	4.6	91.76	−0.065	Cell membrane. Cytoplasm.
NtCML4	Nitab4.5_0002887g0060.1	4	33.33	141	4.45	72.70	−0.650	Cell membrane. Centrosome. Cytoplasm.
NtCML5	Nitab4.5_0001456g0110.1	4	37.96	166	4.06	69.88	−0.530	Cell membrane. Centrosome. Cytoplasm. Nucleus.
NtCML6	Nitab4.5_0002322g0010.1	2	23.13	283	5.19	87.44	−0.363	Endoplasmic reticulum.
NtCML7	Nitab4.5_0000021g0610.1	4	48.65	163	4.39	82.76	−0.425	Vacuole.
NtCML8	Nitab4.5_0000292g0030.1	4	35.00	159	5.24	79.62	−0.469	Cell membrane. Cytoplasm. Nucleus. Spindle pole body. Vacuole.
NtCML9	Nitab4.5_0003562g0010.1	2	38.46	105	5.06	59.52	−1.029	Cell membrane. Cytoplasm.
NtCML10	Nitab4.5_0001824g0030.1	2	43.08	155	9.63	78.58	−0.399	Cytoplasm.
NtCML11	Nitab4.5_0006914g0010.1	4	42.34	150	4.18	78.00	−0.447	Cell membrane. Centrosome. Cytoplasm.
NtCML12	Nitab4.5_0004513g0010.1	4	35.03	241	4.82	92.20	−0.316	Cell membrane. Cytoplasm.
NtCML13	Nitab4.5_0000129g0460.1	4	41.30	186	4.57	72.85	−0.512	Cell membrane.
NtCML14	Nitab4.5_0000221g0010.1	4	40.31	144	4.25	92.92	−0.170	Cytoplasm. Nucleus.
NtCML15	Nitab4.5_0008115g0010.1	2	28.36	89	9.48	63.60	−0.804	Cell membrane. Extracell.
NtCML16	Nitab4.5_0001847g0030.1	2	33.87	89	9.30	72.36	−0.593	Cell membrane. Cytoplasm. Nucleus.
NtCML17	Nitab4.5_0001101g0050.1	4	29.51	102	9.47	82.35	−0.615	Cell membrane. Cytoplasm.

(Continued)

Table 2 (continued)

Name	Gene ID	EF-hands	AtCaM2 (%)	Number of amino acids	pI	Aliphatic index	GRAVY	Subcellular localization
NtCML18	Nitab4.5_0001965g0080.1	4	42.18	160	4.34	96.44	−0.155	Cell membrane. Cytoplasm. Nucleus.
NtCML19	Nitab4.5_0000977g0060.1	4	71.92	147	4.00	86.94	−0.462	Cell membrane. Cytoplasm.
NtCML20	Nitab4.5_0000082g0370.1	4	31.72	219	4.69	69.91	−0.461	Cell membrane.
NtCML21	Nitab4.5_0003855g0030.1	2	36.51	86	4.30	73.84	−0.499	Cell membrane. Cytoplasm.
NtCML22	Nitab4.5_0003855g0020.1	4	43.70	140	5.11	66.86	−0.570	Cell membrane. Cytoplasm. Nucleus.
NtCML23	Nitab4.5_0000036g0500.1	4	40.58	146	4.68	63.49	−0.647	Cell membrane. Centrosome. Cytoplasm. Nucleus.
NtCML24	Nitab4.5_0000036g0480.1	2	40.00	86	4.21	66.98	−0.651	Cell membrane. Cytoplasm. Nucleus.
NtCML25	Nitab4.5_0000212g0050.1	2	46.15	105	4.12	77.14	−0.607	Cell membrane. Cytoplasm.
NtCML26	Nitab4.5_0002016g0050.1	4	48.59	149	4.49	66.24	−0.759	Cell membrane. Nucleus.
NtCML27	Nitab4.5_0000568g0010.1	2	30.08	188	4.78	90.21	−0.031	Cell membrane. Cytoplasm.
NtCML28	Nitab4.5_0002664g0030.1	4	43.88	158	4.71	66.71	−0.478	Centrosome. Spindle pole body. Vacuole.
NtCML29	Nitab4.5_0002664g0060.1	2	50.94	79	4.30	58.10	−0.775	Cytoplasm.
NtCML30	Nitab4.5_0000922g0050.1	4	52.90	185	4.39	72.65	−0.443	Cell membrane.
NtCML31	Nitab4.5_0005658g0030.1	4	35.03	177	4.60	80.34	−0.682	Cell membrane. Centrosome. Cytoplasm.
NtCML32	Nitab4.5_0000038g0320.1	4	43.48	152	4.45	74.93	−0.566	Cell membrane. Centrosome. Cytoplasm. Nucleus.
NtCML33	Nitab4.5_0000457g0360.1	4	42.00	226	4.82	79.78	−0.308	Cell membrane.
NtCML34	Nitab4.5_0000863g0010.1	2	47.87	95	4.10	95.47	−0.279	Cell membrane. Cytoplasm.
NtCML35	Nitab4.5_0001348g0020.1	4	43.08	143	4.52	79.65	−0.528	Cell membrane.

(Continued)

Table 2 (continued)

Name	Gene ID	EF-hands	AtCaM2 (%)	Number of amino acids	pI	Aliphatic index	GRAVY	Subcellular localization
NtCML36	Nitab4.5_0007189g0050.1	2	40.91	196	4.27	84.03	−0.097	Cell membrane. Cytoplasm.
NtCML37	Nitab4.5_0002084g0060.1	4	42.34	150	4.22	75.40	−0.497	Cell membrane.
NtCML38	Nitab4.5_0000529g0080.1	4	44.59	163	4.47	85.15	−0.394	Vacuole.
NtCML39	Nitab4.5_0005031g0050.1	4	35.17	225	4.53	69.78	−0.501	Cell membrane.
NtCML40	Nitab4.5_0000677g0010.1	4	34.48	209	4.70	67.22	−0.542	Cell membrane.
NtCML41	Nitab4.5_0000321g0270.1	4	73.03	155	3.97	82.32	−0.473	Cell membrane. Cytoplasm.
NtCML42	Nitab4.5_0000646g0070.1	4	41.55	161	4.10	102.42	0.009	Cell membrane. Centrosome. Cytoplasm. Nucleus. Spindle pole body.
NtCML43	Nitab4.5_0000686g0100.1	2	32.20	89	9.44	73.48	−0.692	Cell membrane. Cytoplasm. Extracell.
NtCML44	Nitab4.5_0000686g0120.1	2	29.17	89	9.44	67.98	−0.792	Cell membrane. Cytoplasm.
NtCML45	Nitab4.5_0000686g0170.1	2	28.00	89	9.33	70.11	−0.676	Cell membrane. Cytoplasm.
NtCML46	Nitab4.5_0000686g0180.1	2	30.49	89	9.55	59.21	−0.837	Cell membrane. Cytoplasm.
NtCML47	Nitab4.5_0001128g0060.1	2	33.33	220	4.57	78.45	−0.335	Cell membrane. Cytoplasm. Extracell.
NtCML48	Nitab4.5_0001349g0010.1	4	34.64	182	4.45	80.27	−0.653	Cell membrane.
NtCML49	Nitab4.5_0001595g0010.1	4	43.92	163	4.47	85.09	−0.418	Vacuole.
NtCML50	Nitab4.5_0001758g0010.1	2	78.57	99	4.50	85.66	−0.540	Cell membrane. Cytoplasm.
NtCML51	Nitab4.5_0001762g0040.1	4	48.65	191	4.56	86.44	−0.312	Vacuole.
NtCML52	Nitab4.5_0002803g0050.1	4	53.19	189	4.48	84.60	−0.331	Cell membrane. Cytoplasm. Nucleus.
NtCML53	Nitab4.5_0002993g0050.1	4	41.56	202	4.82	79.11	−0.213	Cell membrane. Endoplasmic reticulum.
NtCML54	Nitab4.5_0003007g0040.1	4	40.28	220	5.00	86.09	−0.318	Cell membrane. Nucleus.
NtCML55	Nitab4.5_0003368g0020.1	4	33.33	209	4.62	67.22	−0.514	Cell membrane.

(Continued)

Table 2 (continued)

Name	Gene ID	EF-hands	AtCaM2 (%)	Number of amino acids	pI	Aliphatic index	GRAVY	Subcellular localization
NtCML56	Nitab4.5_0003968g0020.1	4	38.69	164	4.03	70.73	−0.527	Cell membrane. Centrosome. Cytoplasm. Nucleus. Spindle pole body. Vacuole.
NtCML57	Nitab4.5_0004342g0030.1	4	40.69	161	4.14	104.84	0.025	Cell membrane. Centrosome. Cytoplasm. Nucleus. Spindle pole body.
NtCML58	Nitab4.5_0004623g0010.1	2	33.08	198	4.70	85.15	−0.343	Cell membrane. Cytoplasm.
NtCML59	Nitab4.5_0004878g0060.1	4	41.67	160	4.34	93.37	−0.164	Cell membrane. Centrosome. Nucleus.
NtCML60	Nitab4.5_0006002g0020.1	4	43.48	152	4.45	74.93	−0.539	Cell membrane. Cytoplasm. Nucleus.
NtCML61	Nitab4.5_0007144g0030.1	4	43.08	140	4.52	78.57	−0.555	Cell membrane. Centrosome.
NtCML62	Nitab4.5_0007150g0060.1	3	31.78	183	4.83	83.17	−0.537	Cell membrane. Cytoplasm.
NtCML63	Nitab4.5_0008692g0020.1	2	44.62	100	4.12	84.90	−0.420	Cell membrane. Cytoplasm.
NtCML64	Nitab4.5_0009467g0010.1	4	39.26	154	4.26	67.79	−0.532	Cell membrane. Centrosome. Cytoplasm. Nucleus. Spindle pole body. Vacuole.
NtCML65	Nitab4.5_0009593g0010.1	4	43.17	158	4.65	65.44	−0.492	Centrosome. Cytoplasm. Spindle pole body. Vacuole.
NtCML66	Nitab4.5_0009638g0020.1	2	50.94	81	4.34	61.48	−0.763	Cytoplasm.
NtCML67	Nitab4.5_0009710g0010.1	4	48.59	177	4.72	60.17	−0.922	Cell membrane.
NtCML68	Nitab4.5_0009939g0030.1	4	47.89	149	4.48	65.57	−0.776	Cell membrane. Nucleus.
NtCML69	Nitab4.5_0010741g0010.1	4	40.58	210	4.96	69.14	−0.517	Cell membrane.

(Continued)

Table 2 (continued)

Name	Gene ID	EF-hands	AtCaM2 (%)	Number of amino acids	pI	Aliphatic index	GRAVY	Subcellular localization
NtCML70	Nitab4.5_0011320g0020.1	2	36.36	165	4.28	70.91	−0.618	Cell membrane. Cytoplasm.
NtCML71	Nitab4.5_0011970g0010.1	4	39.26	157	4.26	66.50	−0.518	Cell membrane. Centrosome. Cytoplasm. Nucleus. Spindle pole body. Vacuole.
NtCML72	Nitab4.5_0012187g0010.1	4	33.79	219	4.59	68.58	−0.498	Cell membrane.
NtCML73	Nitab4.5_0012644g0010.1	4	76.87	150	4.04	83.80	−0.499	Cell membrane. Cytoplasm.
NtCML74	Nitab4.5_0013265g0020.1	4	35.53	182	4.65	82.42	−0.600	Cell membrane. Centrosome. Cytoplasm.
NtCML75	Nitab4.5_0024537g0010.1	4	52.17	179	4.36	70.73	−0.471	Cell membrane. Nucleus.

3.2 Gene Structure and Conserved Motif Analysis of the NtCMLs in Tobacco

Motifs are important in identifying Transcription Factor Binding Sites, which helps understand the mechanisms that regulate gene expression [32]. The MEME tool was employed to locate conserved motifs for a more thorough examination of the NtCML proteins (Fig. 1A). Motif 1 and motif 2 were present in all 75 *NtCMLs* family members. Motifs 3, 4, 5, 6, and 8 are most commonly observed in the N-terminus and motifs 1, 2, and 7 are present in the C-terminus. Some paralogous proteins contained different motifs, such as *NtCML14* and *NtCML54*, *NtCML12* and *NtCML31*, *NtCML6* and *NtCML9, NtCML41* and *NtCML50*, while *NtCML18* and *NtCML59*, *NtCML38* and *NtCML49*, *NtCML48* and *NtCML74*, *NtCML11* and *NtCML37*, *NtCML40* and *NtCML55* had the same motif. Motif 9 is exclusively in *NtCML3* and *NtCML27*.

Most NtCMLs belong to the PTZ00184 superfamily, which is also known as the EF-hand protein superfamily (Fig. 1B), Introns can increase transcript levels, and exons as enhancers are crucial in protein synthesis [33,34]. We analyzed the exon-intron structure of *NtCMLs* genes to describe their conservation and differences (Fig. 1C). According to the findings, NtCMLs contain between one and eight exons, and the most of their members lack introns.

3.3 Phylogenetic Analysis of NtCMLs Proteins

To further comprehend the links between these compounds, phylogenetic analysis was performed using the recovered tobacco protein sequences, we constructed the phylogenetic tree with 125 NtCMLs protein sequences, including 75 sequences from tobacco (*NtCMLs*) and 50 from Arabidopsis (*AtCMLs*). These *NtCMLs* protein were classified into Five subgroups (Group I–Group V) (Fig. 2). The smallest subgroup was Group I which consisted of 4 CMLs (3 *AtCMLs* and 1 *NtCMLs*). The largest subgroup was Group V which consisted of 49 CMLs (19 *AtCMLs* and 30 *NtCMLs*). Group II included 18 CMLs members (3 *AtCMLs* and 15 *NtCMLs*). The Group III included 17 CML members (7 *AtCMLs* and 10 *NtCMLs*). The

Group IV included 37 CMLs members (18 *AtCMLs* and 19 *NtCMLs*). Furthermore, the majority of NtCML was homologous to Arabidopsis. The findings suggest that CMLs are conserved across all plant species.

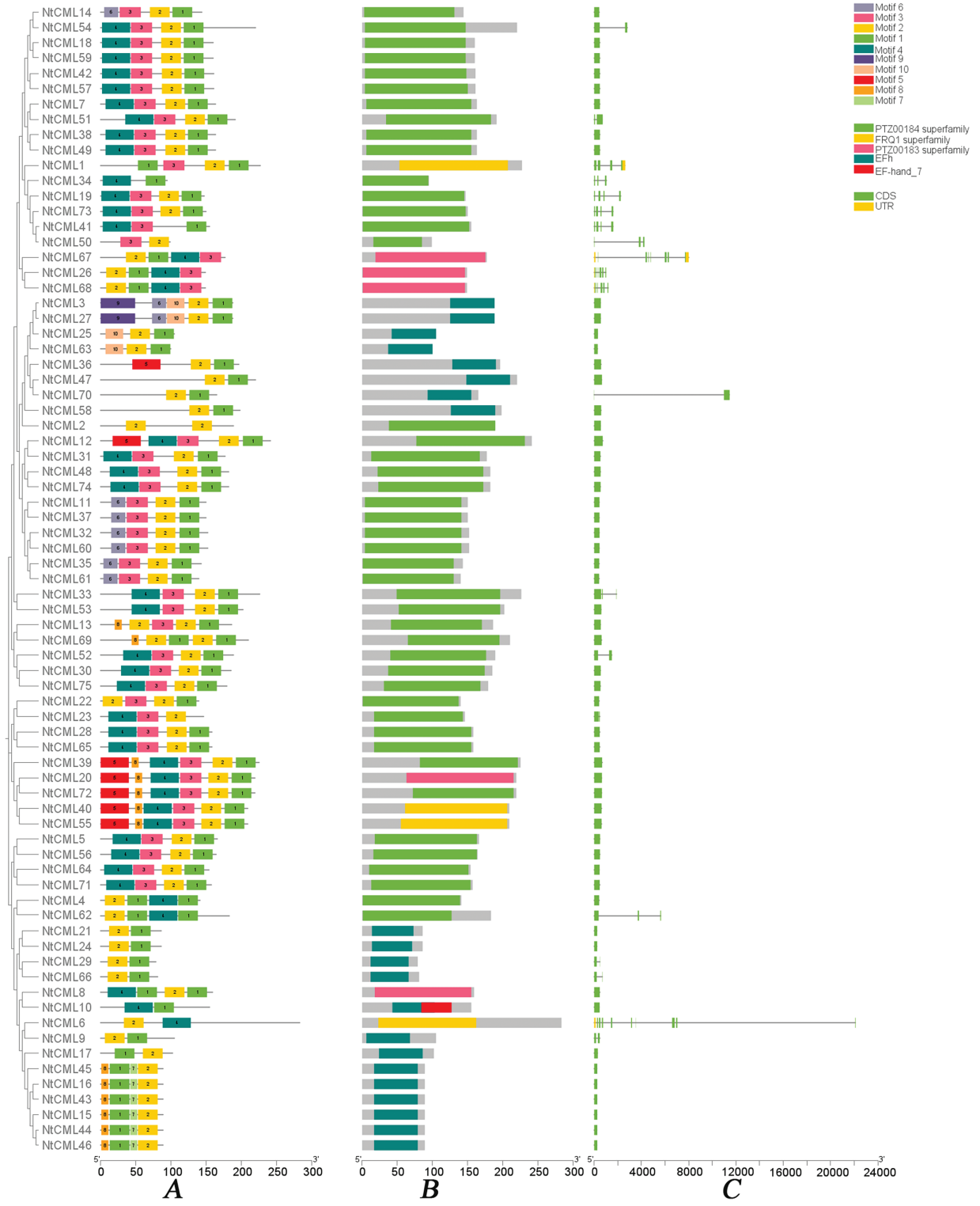

Figure 1: (A) Motif structure of NtCMLs; (B) Domain of NtCMLs; (C) The gene structure of NtCMLs

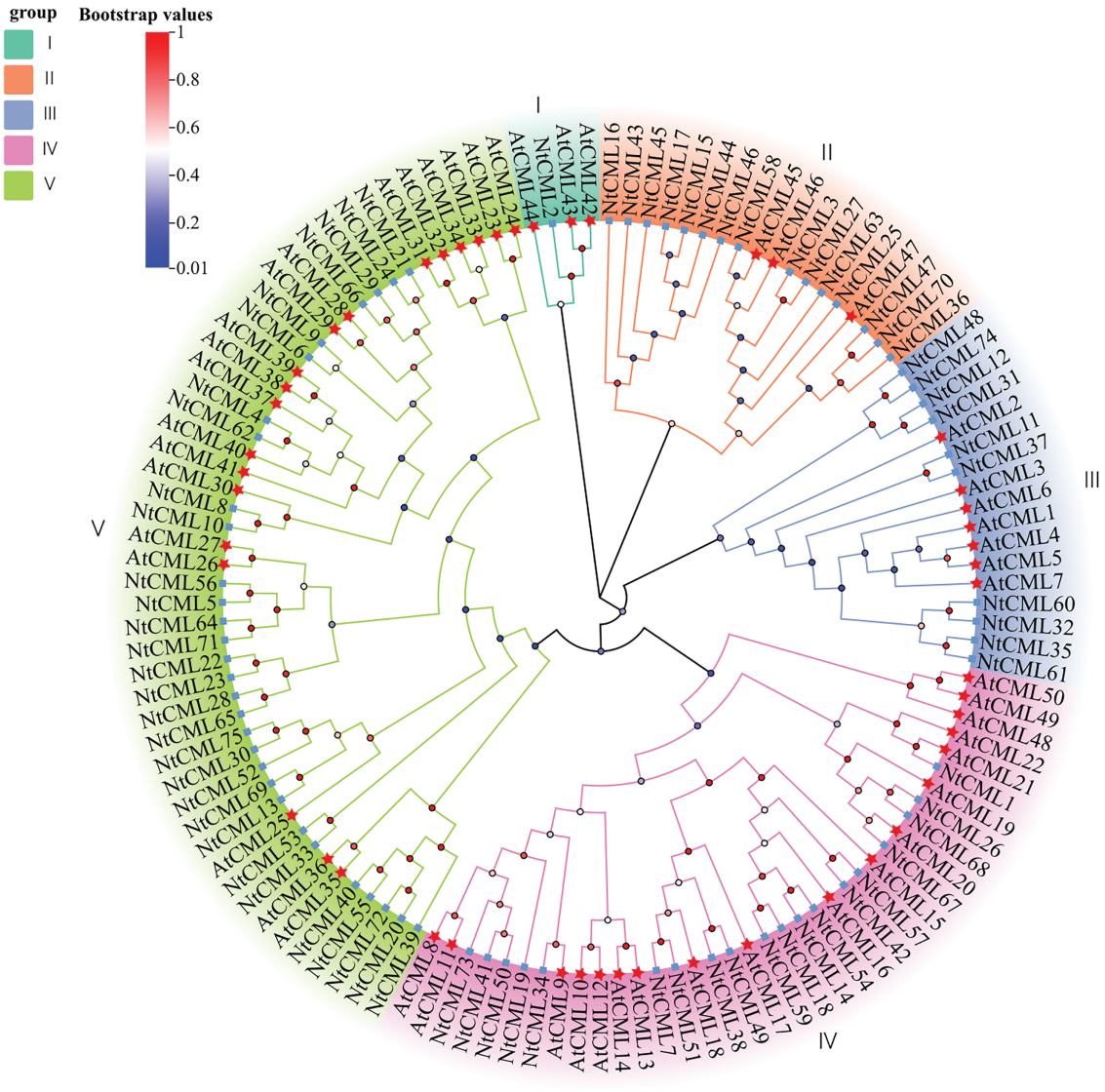

Figure 2: Phylogenetic tree of NtCMLs proteins in tobacco and Arabidopsis

3.4 Cis-Acting Elements Analysis in Promoter Regions of NtCMLs Genes

Gene expression is significantly influenced by parallel trans-regulatory factors and cis-elements. To better understand transcriptional and functional control of the *NtCMLs* genes, the extraction of 2000-bp upstream sequences of the NtCMLs coding areas was performed by analyzing cis-acting elements (Fig. 3). The primary cis-acting factors linked to plant growth and development, biotic and abiotic stressors, light, and hormones (Fig. 4). Among the elements that respond to plant hormones are abscisic acid (ABRE), methyl jasmonate (CGTCA-motif, TGACG-motif), ethylene (ERE), and salicylic acid (TCA). Abiotic and biotic stressors such as low temperature (LTR), drought (MBS), anaerobic (ARE), and W-box are associated with the element type. Other components of the stress response have also been identified, such as the light element G-box and the trauma response element WUN-motif. The cis-element analysis highlighted the regulatory complexity of the *NtCMLs* gene family, suggesting that the most of NtCMLs genes are essential for plant development and growth, as well as hormone and stress responses.

Figure 3: Cis-acting elements analysis of NtCMLs

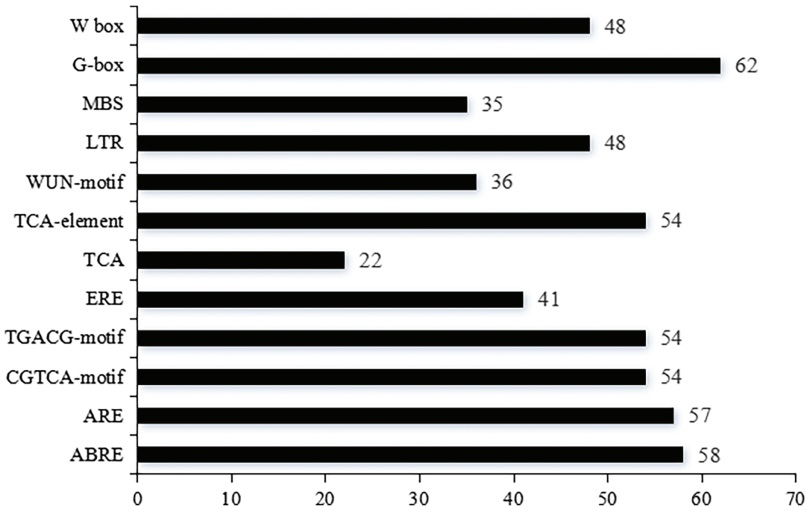

Figure 4: Number of NtCMLs genes containing cis-acting elements

3.5 Chromosomal Location and Synteny Analysis of NtCMLs Genes

The protein sequences of the discovered NtCMLs were used to determine their chromosomal locations. The findings revealed that tobacco has 18 chromosomes, which contain 40 *NtCMLs* genes (Fig. 5). Particularly, chromosome 17 has the most genes, followed by chromosome 12, which contains five *NtCML* genes, and chromosome 4 contains four NtCML genes. Ten Chromosomes contain only one *NtCML*s genes (Chromosomes 1, 2, 5, 6, 8, 9, 13, 14, 15, 24). The results demonstrated that *NtCMLs* were randomly distributed across different chromosomes.

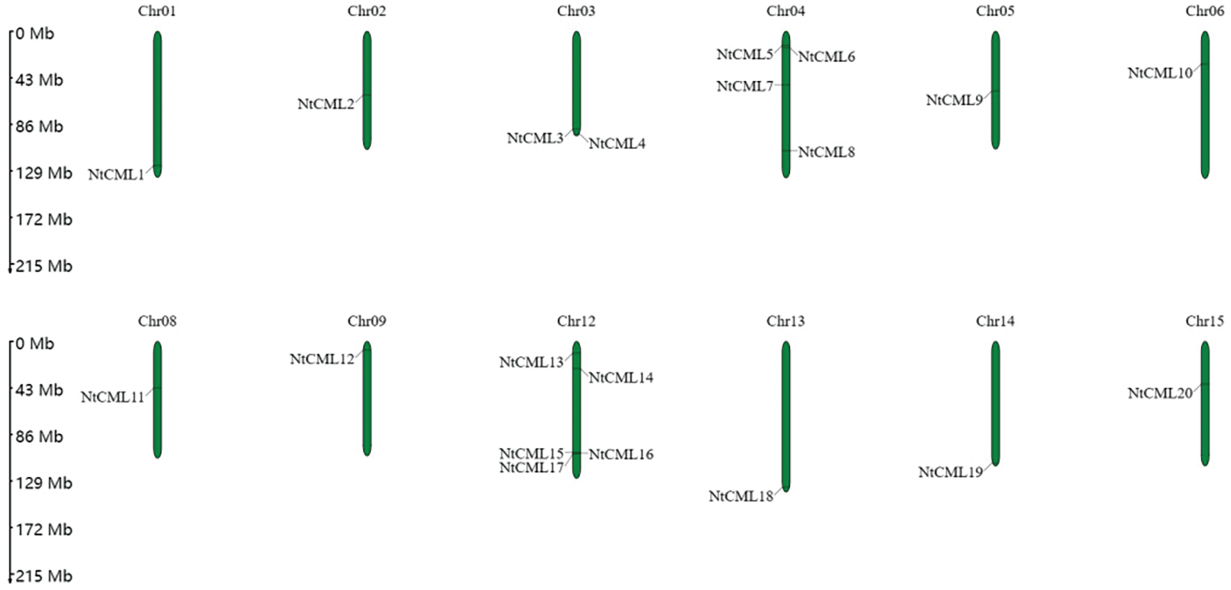

Figure 5: (Continued)

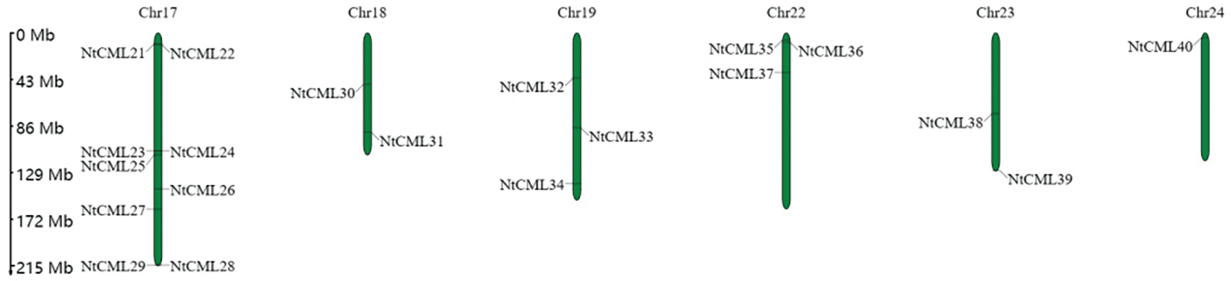

Figure 5: Chromosome distribution of *NtCMLs* genes in *Nicotiana tabacum*

To determine the *NtCMLs* gene duplication of the *NtCMLs* genes, the segmental duplication events in the *NtCMLs* gene family were conducted. 12 *NtCMLs* genes were predicted to be segmentally duplicated on chromosomes 3, 8, 12, 15, 17, 19 and 22. The tobacco genome include seven segmental duplicated gene pairs and two tandemly duplicated gene pairs (Fig. 6). These duplicate genes are probably caused by intra- or inter-chromosomal segmental duplication of other genes.

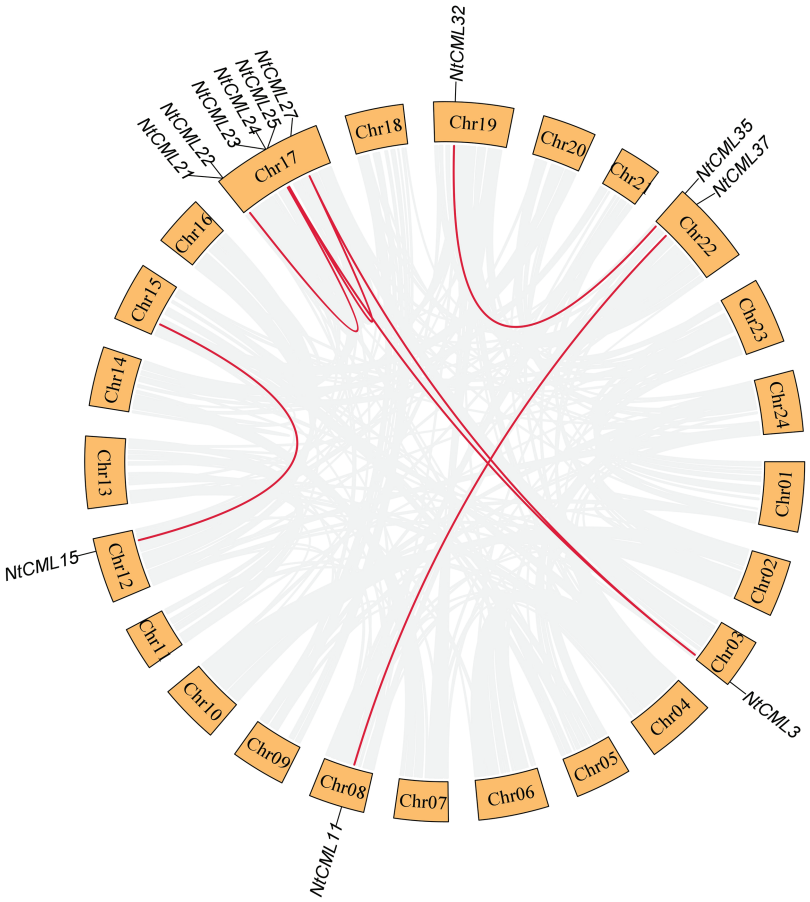

Figure 6: Interchromosomal relationshipsf of NtCMLs in *Nicotiana tabacum*

A comparative analysis of the genomes of tobacco and Arabidopsis was conducted to gain a better understanding of the evolution of *NtCMLs* genes (Fig. 7). 22 pairs of orthologous genes were present between tobacco and *Arabidopsis*, there was one-to-many or many-to-one collinearity between the *NtCMLs* and the *AtCMLs* (Table 3). For instance, four genes (*NtCML5*, *MtCML28*, *NtCML25, NtCML26*) had two homologous genes in *Arabidopsis*, while one gene (*NtCML27*) had three orthologous genes. The existence of ancient gene pairs during the divergence of Arabidopsis and tobacco has been demonstrated, and their functions may be similar.

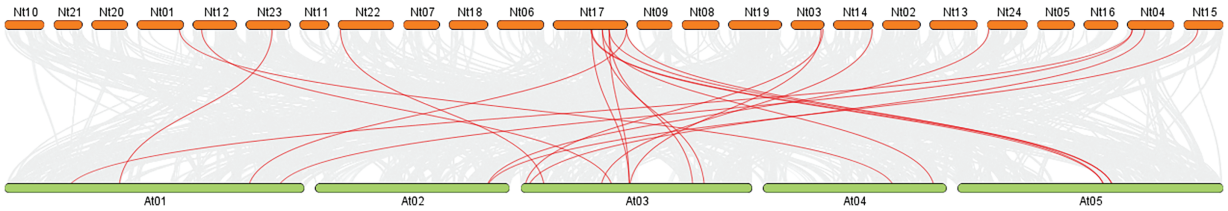

Figure 7: Synteny analysis of CMLs in *Nicotiana tabacum* and Arabidopsis

Table 3: Colinear gene pairs of *Nicotiana tabacum* and *Arabidopsis thaliana*

Arabidopsis chromosomes	Arabidopsis login number	Arabidopsis ID	Tobacco chromosome	Tobacco entry number	Tobacco ID
At01	AT1G18210.2	*AtCML27*	Nt04	Nitab4.5_0001456g0110.1	*NtCML5*
At01	AT1G73630.1	*AtCML26*	Nt04	Nitab4.5_0001456g0110.1	*NtCML5*
At01	AT1G66400.1	*AtCML23*	Nt17	Nitab4.5_0002664g0030.1	*NtCML28*
At01	AT1G32250.1	*AtCML17*	Nt23	Nitab4.5_0000529g0080.1	*NtCML38*
At02	AT2G41410.1	*AtCML35*	Nt15	Nitab4.5_0000082g0370.1	*NtCML20*
At02	AT2G41410.1	*AtCML35*	Nt24	Nitab4.5_0000677g0010.1	*NtCML40*
At03	AT3G29000.1	*AtCML45*	Nt03	Nitab4.5_0002980g0030.1	*NtCML3*
At03	AT3G01830.1	*AtCML40*	Nt03	Nitab4.5_0002887g0060.1	*NtCML4*
At03	AT3G03000.1	*AtCML18*	Nt04	Nitab4.5_0000021g0610.1	*NtCML7*
At03	AT3G25600.1	*AtCML16*	Nt12	Nitab4.5_0000221g0010.1	*NtCML14*
At03	AT3G22930.1	*AtCML11*	Nt14	Nitab4.5_0000977g0060.1	*NtCML19*
At03	AT3G29000.1	*AtCML45*	Nt17	Nitab4.5_0000568g0010.1	*NtCML27*
At03	AT3G29000.1	*AtCML45*	Nt17	Nitab4.5_0000212g0050.1	*NtCML25*
At03	AT3G50360.1	*AtCML20*	Nt17	Nitab4.5_0002016g0050.1	*NtCML26*
At03	AT3G47480.1	*AtCML47*	Nt17	Nitab4.5_0000568g0010.1	*NtCML27*
At03	AT3G07490.1	*AtCML3*	Nt22	Nitab4.5_0001348g0020.1	*NtCML35*
At04	AT4G26470.3	*AtCML21*	Nt01	Nitab4.5_0001622g0090.1	*NtCML1*
At04	AT4G37010.2	*AtCML19*	Nt17	Nitab4.5_0002016g0050.1	*NtCML26*
At05	AT5G37770.1	*AtCML24*	Nt17	Nitab4.5_0002664g0030.1	*NtCML28*
At05	AT5G37770.1	*AtCML24*	Nt17	Nitab4.5_0000036g0500.1	*NtCML23*
At05	AT5G39670.1	*AtCML46*	Nt17	Nitab4.5_0000568g0010.1	*NtCML27*
At05	AT5G39670.1	*AtCML46*	Nt17	Nitab4.5_0000212g0050.1	*NtCML25*

3.6 Spatio-Temporal Expression Patterns of NtCMLs Genes in Different Tissues

To determine the possible roles of *NtCMLs*, the public transcription data for several tobacco K326 tissues, including seed, shoot, flower, stem, and root was downloaded. The different expression patterns in most of the 19 tissues and developmental stages were analyzed (Fig. 8). Some *NtCMLs* genes exhibited a tissue-specific expression pattern. Eight NtCMLs genes (*NtCML19, NtCML41, NtCML50, NtCML73, NtCML6, NtCML10, NtCML38, NtCML49*) were highly expressed in the seed, *NtCML21, NtCML24, NtCML66, NtCML8, and NtCML29* were highly expressed in flowers, three *NtCMLs* genes (*NtCML70, NtCML36, NtCML47*) were highly expressed in young shoot. Furthermore, some NtCMLs genes showed similar expression patterns, including *NtCML75* and *NtCML30*, *NtCML41* and *NtCML50*, *NtCML62* and *NtCML4*, *NtCML54* and *NtCML14*. The findings suggested that these genes may have similar functions in plant growth and development.

3.7 Expression of the Selected 10 NtCMLs Genes in Response to Cold Acclimation

Ten *NtCMLs* genes were examined for their expression in response to cold acclimation (Fig. 9). The transcript levels of *NtCML8*, *NtCML18*, *NtCML12*, *NtCML23*, *NtCML27*, and *NtCML2*8 were significantly upregulated following exposure to cold conditions. In contrast, transcripts for *NtCML22* and *NtCML53* showed a significant reduction. However, no significant changes were observed in the expression of *NtCML30* and *NtCML3* under cold stress when compared with the other genes. After 7 days of cold acclimation, we found that *NtCML3*, *NtCML12*, *NtCML18*, *NtCML27*, and *NtCML28* were still significantly upregulated, while *NtCML8* and *NtCML30* were significantly down-regulated. However, *NtCML22*, *NtCML23*, and *NtCML53* had no significant change. However, when exposed to low-temperature stress once more, *NtCML3*, *NtCML12*, *NtCML22*, *NtCML18*, *NtCML23*, *NtCML27*, *NtCML28*, *NtCML30*, and *NtCML53* significantly upregulated. These findings imply that the *NtCMLs* gene has distinct functions in the cold acclimation recovery process.

3.8 Protein Interaction Network Prediction

In this study, 75 NtCMLs proteins were analyzed using STRING to predict the protein interaction network in tobacco (Fig. 10). 13 NtCMLs proteins were identified as participating in the interaction network, and four CMLs proteins exhibited correlations with more than four other CMLs. Notably, CML46 was associated with ten NtCMLs proteins. The protein-protein associations suggested that some NtCMLs proteins are likely co-expressed based on findings from Arabidopsis research. NtCML4, NtCML59, and NtCML2 demonstrated a close protein interaction and exhibited potential co-expression and co-occurrence patterns. The analysis of the protein interaction network indicated that NtCMLs regulate downstream gene expression through interactions with other proteins, thereby providing a valuable resource for further research.

4 Discussion

Numerous aspects of plant growth and development, including stress responses, are significantly influenced by calcium ions (Ca^{2+}). Calmodulin-like proteins (CMLs), which act as calcium ion sensors, play a crucial role in cellular signaling networks by regulating a wide range of targets [1]. Using bioinformatics techniques, 75 NtCMLs genes were found in the tobacco genome in this study. In comparison to *Arabidopsis thaliana* (50 *CMLs*) [12], rice (32 *CMLs*) [13] and tomato (52 *CMLs*) [14], tobacco possesses a greater number of *CMLs* genes, This discrepancy may be attributed to the allotetraploid nature of tobacco. A comprehensive bioinformatics analysis revealed significant variations in amino acid number, pI, aliphatic index, and instability index between different NtCMLs proteins. The majority of NtCMLs (85%) were acidic, consistent with the performance of CMLs family members in cucumber [10], apple [4], and tomato [14]. Previous studies have demonstrated that CaMs and CMLs exhibit high preservation properties. In the Arabidopsis [12], cucumber [10], and papaya [35], the CMLs

shared the 16.1%~74.5%, 24%~77%, and 22.4%~88.1% identity with *AtCaM2*. In this study, We decided to use 16%~80% amino acid similarity as the selection criterion.

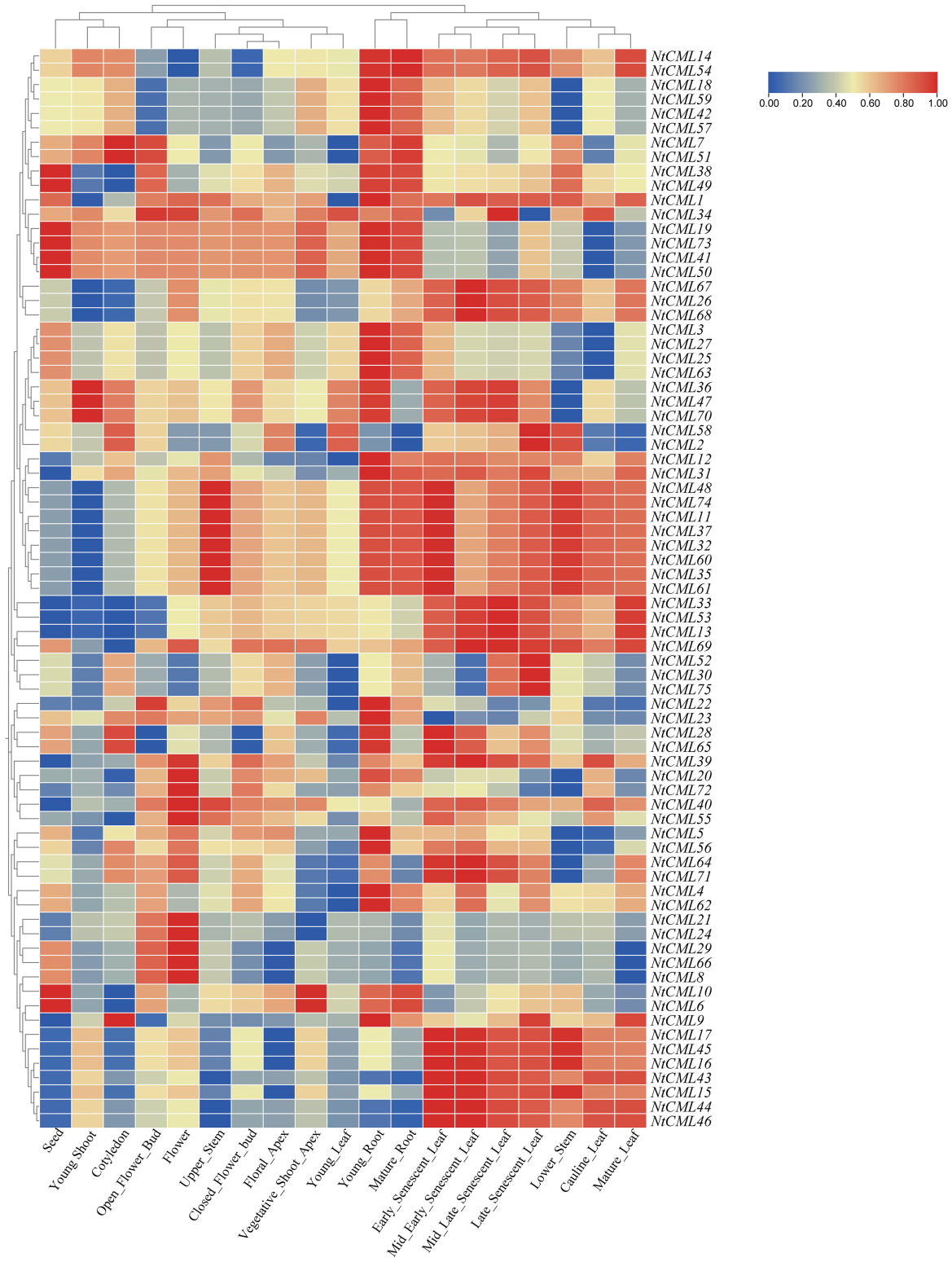

Figure 8: The relative expression patterns of NtCMLs genes in different tissues

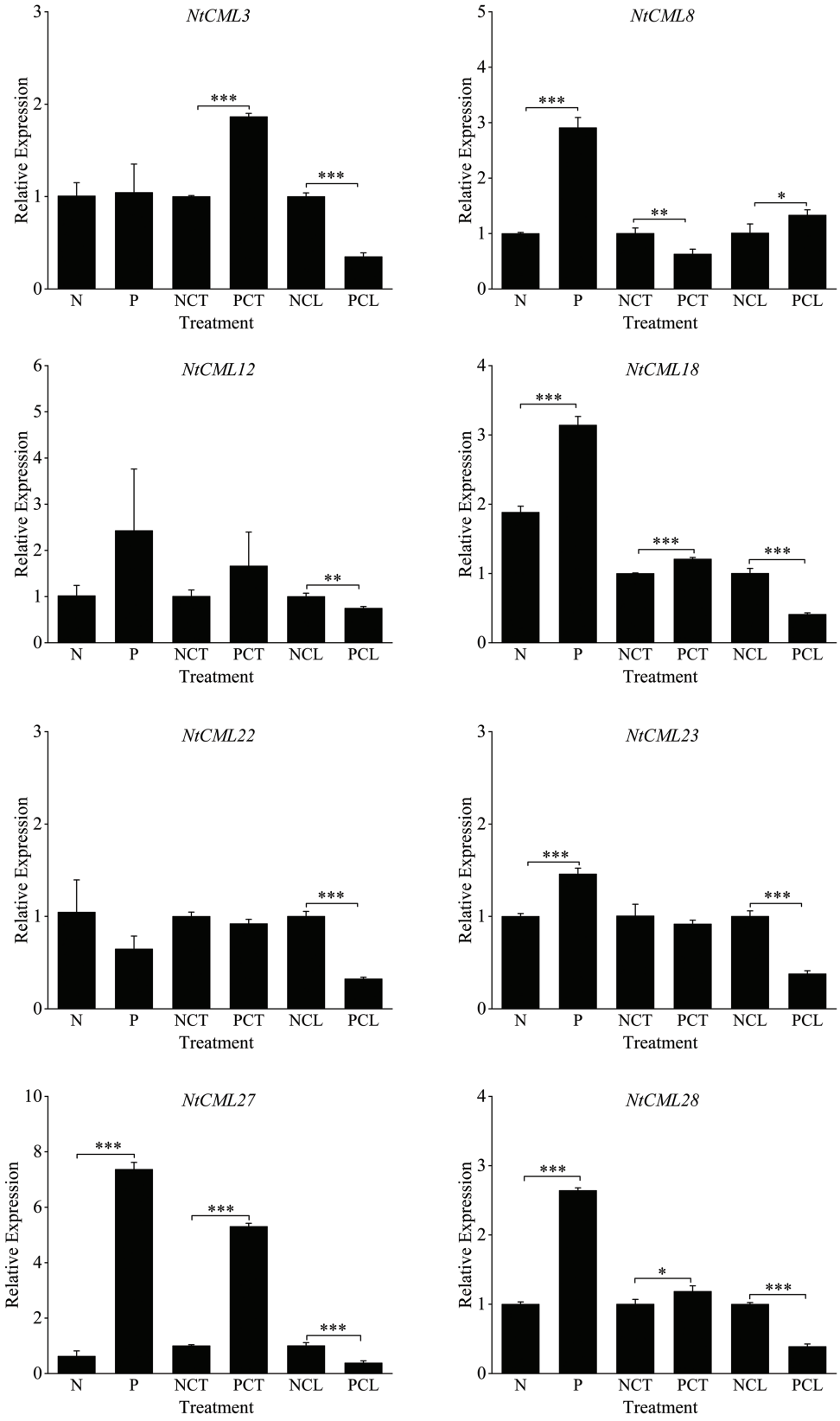

Figure 9: (Continued)

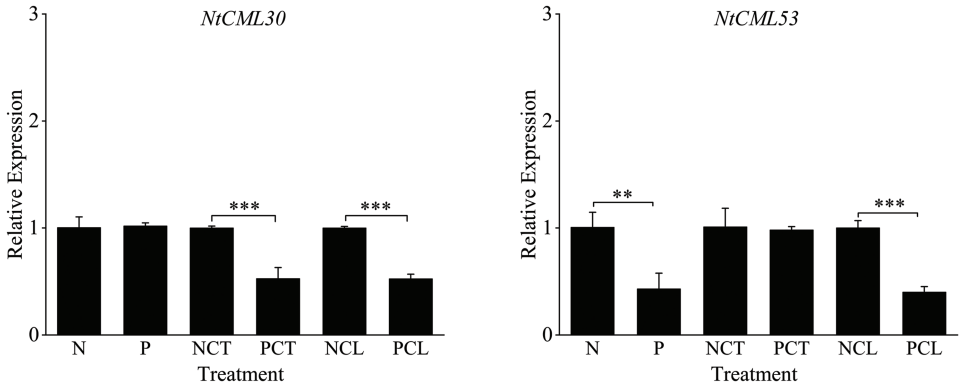

Figure 9: Levels of NtCMLs family members' relative expression during cold adaptation. Each sample underwent three separate experiments. *, **, *** indicate significant difference at 0.05, 0.01.0.001

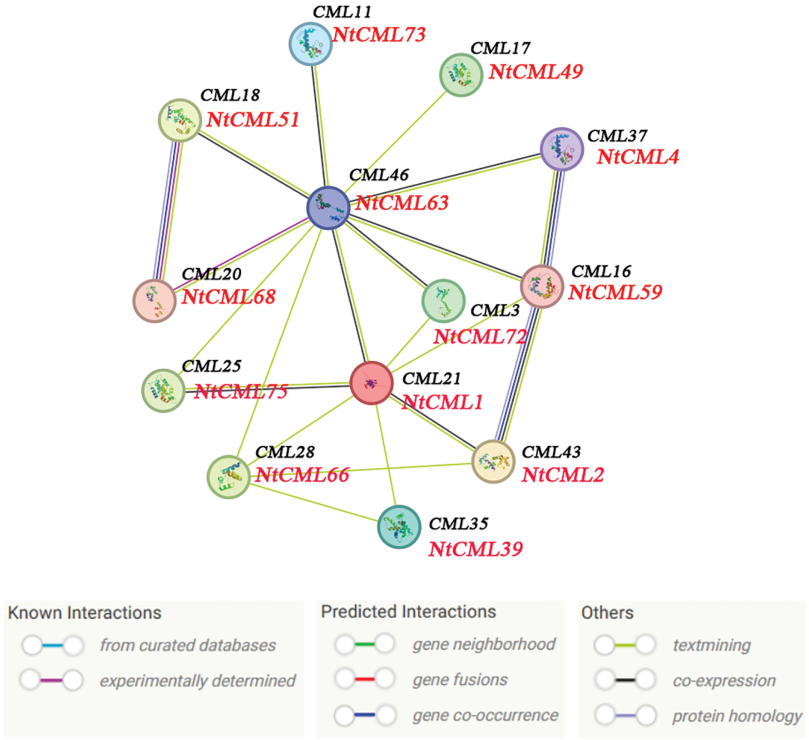

Figure 10: Protein interaction network of CMLs proteins. The homologous genes from tobacc and Arabidopsis are in red and black, respectively

Gene location analysis showed that the 40 *NtCMLs* genes were not evenly dispersed across the tobacco's 18 chromosomes, with the highest concentration being on chromosome 17. Calcium ion binding may be affected by the number of CMLs proteins that have conserved structural domains of the EF hands [10]. In Arabidopsis, which usually has 2~6 EF-hands structural domains [12], while tobacco possesses 2~4, which is consistent with cabbage [36] and common beans [37]. Previous studies have demonstrated that the majority of *CMLs* lack introns [38]. In tobacco, most *NtCMLs* genes lack introns. This instance aligned with grape [11], cucumber [10], and cabbage [36], proving that the gene structure of CMLs has been preserved through plant evolution. The conserved motif results demonstrated that both motif 1 and

motif 2 were present in all members of the NtCMLs family. The diverse NtCMLs were indicated by the different conserved motifs.

A phylogenetic evolutionary tree analysis of tobacco and Arabidopsis reveals that the 125 CMLs proteins are divided into five groups, with at least one tobacco and Arabidopsis CMLs protein in each group, while four subgroups are present in *Solanum pennellii* [39] and seven are present in cucumber [10]. Additionally, the analysis indicates that many NtCMLs proteins are homologous to Arabidopsis. Gene duplication is an essential process for organisms to acquire new genes, which leads to genetic novelty, and has resulted in numerous new gene functions that have greatly advanced biological evolution [40]. Seven segmental duplicated gene pairs and two tandemly duplicated gene pairs were found in the tobacco genome during our investigation, while two tandemly duplicated gene pairs and three segmentally duplicated gene pairs in the cucumber genome [10]. 22 pairs of orthologous genes were present between tobacco and Arabidopsis, while five collinear gene pairs between cucumber and Arabidopsis [10]. It suggests that plants are highly conserved during evolution.

Gene expression regulates the growth, development, and adaptation of plants. This process is dependent on promoters, which are the cis-acting regions that initiate transcription [41]. This research revealed that the promoters of CMLs in tobacco were enriched with cis-acting elements that were linked to biological stress responses and plant hormones. Some specific cis-elements (ABRE, CGTCA-motif, LTR, MBS, ERE, and WUN-motif, among others) were in the promoter regions of *NtCMLs* genes. Similar to the reports of *Medicago truncatula* [2], apple [4], and Chrysanthemum seticuspe [15]. Previous studies have demonstrated that some *CMLs* genes are involved in hormonal or abiotic stress responses. *CML9*, *CML24*, showed response to ABA and salt stresses [19,21]. In this study, *NtCML21*, *NtCML24* and *CML9*, *CML24* belong to the same subfroup, and *NtCML21*, *NtCML24* include ABA cis-acting elements, indicating that they could be involved in ABA response. *CML37*, *CML38*, *CML39*, *MtCML40*, and *MpCML40* showed extensive responses to salt stress [27,28,31], *CML39* is involved in the growth and development of seeds [18]. These previous studies have demonstrated that NtCMLs genes are crucial for plant growth, development, and adaptation to adverse conditions.

Examining the spatiotemporal differential expression patterns of NtCMLs in various organs may help to better understand their potential roles in tobacco growth and development. In this study, the *NtCMLs* gene family displayed a wide range of expression patterns in diverse tissues and organs during different phases of development. *CML23* and *CML24* are connected with flower development, our findings indicate that *NtCML21*, *NtCML24*, and *NtCML66* are substantially upregulated in flowers and belong to the same subgroup, implying that they may be involved in flowering and fruit growth [26,41]. *NtCML59*, *NtCML57*, and *NtCML42* genes were highly expressed in roots, which were presumed to be involved in the process of root growth and development. In conclusion, the spatiotemporal expression pattern of 75 *NtCMLs* genes demonstrated a tissue specificity.

The physiological and biochemical functions of plants can be regulated by short-term exposure to non-lethal cold temperatures, which can enhance their resistance to cold stress. This phenomenon is known as cold acclimation (CA) [42]. Plants have developed the CA mechanism to reduce the negative effects of cold stress, which is recognized as a common process that enables many temperate species to develop cold tolerance and resistance to freezing [43]. During CA treatment, elevated expression levels of calmodulin phosphatase b-like proteins (CBLs), calmodulin-interacting protein kinases (CIPKs), calmodulin-like proteins (CMLs), calcium-dependent protein kinases (CDPKs) were observed [44]. The results of the study indicate that *NtCML12*, *NtCML18*, *NtCML27*, and *NtCML28* were significantly upregulated during the CA treatment. Conversely, the majority of *NtCMLs* genes were significantly down-regulated following re-exposure to low-temperature stress, indicating that the plant may have accumulated certain substances during the recovery period to enhance its cold tolerance. These findings

make it easier to investigate the role of *NtCML* genes during CA in tobacco. In conclusion, NtCMLs are connected to cold stress, and earlier research has demonstrated that some genes in plants like *Medicago truncatula* [28] and *Solanum lycopersicum* [14] are similarly impacted by cold stress. During this investigation, it was discovered that *NtCML27* experienced a significant upregulation in response to cold stress and was chosen as a candidate gene to be confirmed in the next phase.

Acknowledgement: We are grateful to the Chiplot and Tbtools software creators. We also want to express our gratitude to the entire crew for their assistance during the trial.

Funding Statement: This study was supported by College of Agronomy and Biotechnology, Southwest University (4412200577).

Author Contributions: Study conception and design: Mengjie Xu, Anbin Wang; data collection: Mengjie Xu, Anbin Wang, Tonghong Zuo; analysis and interpretation of data: Mengjie Xu, Anbin Wang, Tonghong Zuo, Hecui Zhang, Zhihao Hu; draft manuscript preparation: Mengjie Xu; revised the manuscript: Liquan Zhu, Hecui Zhang. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author, L. Q. Z., upon reasonable request.

Ethics Approval: Not applicable.

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

References

1. Perochon A, Aldon D, Galaud JP, Ranty B. Calmodulin and calmodulin-like proteins in plant calcium signaling. Biochimie. 2011;93(12):2048–53. doi:10.1016/j.biochi.2011.07.012.
2. Sun Q, Yu S, Guo Z. Calmodulin-Like (CML) gene family in medicago truncatula: genome-wide identification, characterization and expression analysis. Int J Mol Sci. 2020;21(19):7142. doi:10.3390/ijms21197142.
3. Wang C, Luan S. Calcium homeostasis and signaling in plant immunity. Curr Opin Plant Biol. 2023;77:102485.
4. Li C, Meng D, Zhang J, Cheng L. Genome-wide identification and expression analysis of calmodulin and calmodulin-like genes in apple (Malus × domestica). Plant Physiol Biochem. 2019;139:600–12. doi:10.1016/j.plaphy.2019.04.014.
5. Ma X, Li QH, Yu YN, Qiao YM, Haq SU, Gong ZH. The CBL-CIPK pathway in plant response to stress signals. Int J Mol Sci. 2020;21(16):5668. doi:10.3390/ijms21165668.
6. Virdi AS, Singh S, Singh P. Abiotic stress responses in plants: roles of calmodulin-regulated proteins. Front Plant Sci. 2015;6:809.
7. Tong T, Li Q, Jiang W, Chen G, Xue D, Deng F, et al. Molecular evolution of calcium signaling and transport in plant adaptation to abiotic stress. Int J Mol Sci. 2021;22(22):12308. doi:10.3390/ijms222212308.
8. Aldon D, Mbengue M, Mazars C, Galaud JP. Calcium signalling in plant biotic interactions. Int J Mol Sci. 2018;19(3):665. doi:10.3390/ijms19030665.
9. Jessica LG, Michael PW, Hans JV. Structures and metal-ion-binding properties of the Ca^{2+}-binding helix-loop–helix EF-hand motifs. Biochem J. 2007;405(2):199–221. doi:10.1042/BJ20070255.
10. Liu Y, Yin F, Liao L, Shuai L. Genome-wide identification and expression analysis of calmodulin-like proteins in cucumber. PeerJ. 2023;11:14637. doi:10.7717/peerj.14637.
11. Vandelle E, Vannozzi A, Wong D, Danzi D, Digby AM, Dal Santo S, et al. Identification, characterization, and expression analysis of calmodulin and calmodulin-like genes in grapevine (*Vitis vinifera*) reveal likely roles in stress responses. Plant Physiol Biochem. 2018;129:221–37. doi:10.1016/j.plaphy.2018.06.003.
12. McCormack E, Braam J. Calmodulins and related potential calcium sensors of Arabidopsis. New Phytol. 2003;59:585–98.

13. Boonburapong B, Buaboocha T. Genome-wide identification and analyses of the rice calmodulin and related potential calcium sensor proteins. BMC Plant Biol. 2007;7:4. doi:10.1186/1471-2229-7-4.
14. Munir S, Khan MR, Song J, Munir S, Zhang Y, Ye Z, et al. Genome-wide identification, characterization and expression analysis of calmodulin-like (CML) proteins in tomato (*Solanum lycopersicum*). Plant Physiol Biochem. 2016;102:167–79. doi:10.1016/j.plaphy.2016.02.020.
15. Fu M, Wu C, Li X, Ding X, Guo F. Genome-wide identification and expression analysis of CsCaM/CML gene family in response to low-temperature and salt stresses in *Chrysanthemum seticuspe*. Plants. 2022;11(13):1760. doi:10.3390/plants11131760.
16. Cai K, Kuang L, Yue W, Xie S, Xia X, Zhang G, et al. Calmodulin and calmodulin-like gene family in barley: identification, characterization and expression analyses. Front Plant Sci. 2022;13:964888. doi:10.3389/fpls.2022.964888.
17. Zeng H, Xu L, Singh A, Wang H, Du L. Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses. Front Plant Sci. 2015;6:600.
18. Midhat U, Ting MKY, Teresinski HJ, Snedden WA. The calmodulin-like protein, *CML39*, is involved in regulating seed development, germination, and fruit development in Arabidopsis. Plant Mol Biol. 2018;96:375–92. doi:10.1007/s11103-018-0703-3.
19. Yang X, Wang SS, Wang M, Qiao Z, Bao CC, Zhang W. Arabidopsis thaliana calmodulin-like protein *CML24* regulates pollen tube growth by modulating the actin cytoskeleton and controlling the cytosolic Ca^{2+} concentration. Plant Mol Biol. 2014;86:225–36. doi:10.1007/s11103-014-0220-y.
20. Wang SS, Diao WZ, Yang X, Qiao Z, Wang M, Biswa RA, et al. Arabidopsis thaliana *CML25* mediates the Ca^{2+} regulation of K^+ transmembrane trafficking during pollen germination and tube elongation. Plant Cell Environ. 2015;38(11):2372–86. doi:10.1111/pce.v38.11.
21. Leba LJ, Cheval C, Ortiz-Martin I, Ranty B, Beuzon CR, Galaud JP, et al. *CML9*, an Arabidopsis calmodulin-like protein,contributes to plant innate immunity through a flagellin-dependentsignalling pathway. Plant J. 2012;71(6):976–89. doi:10.1111/tpj.2012.71.issue-6.
22. Xu B, Cheval C, Laohavisit A, Hocking B, Chiasson D, Tjelvar SG, et al. Calmodulin-like protein regulates plasmodesmal closure during bacterial immune responses. New Phytol. 2017;215(1):77–84. doi:10.1111/nph.2017.215.issue-1.
23. Zhu XY, Robe E, Jomat L, Aldon D, Mazars C. *CML8*, an Arabidopsis calmodulin-like protein, plays a role in Pseudomonas syringae plant immunity. Plant Cell Physiol. 2017;58(2):307–19.
24. Magnan F, Ranty B, Charpenteau M, Sotta B, Galaud JP, Aldon D. Mutations in *AtCML9*, a calmodulin-like protein from Arabidopsis thaliana, alter plant responses to abiotic stress and abscisic acid. Plant J. 2008;56(4):575–89. doi:10.1111/tpj.2008.56.issue-4.
25. Wu X, Qiao Z, Liu H, Acharya BR, Li C, Zhang W. *CML20*, an Arabidopsis Calmodulin-like protein, negatively regulates guard cell ABA signaling and drought stress tolerance. Front Plant Sci. 2017;8:824. doi:10.3389/fpls.2017.00824.
26. Delk NA, Johnson KA, Chowdhury NI, Braam J. *CML24*, regulated in expression by diverse stimuli, encodes a potential Ca^{2+} sensor that functions in responses to abscisic acid, daylength, and ion stress. Plant Physiol. 2005;139:240–53. doi:10.1104/pp.105.062612.
27. Vanderbeld B, Snedden WA. Developmental and stimulus-induced expression patterns of Arabidopsis calmodulin-like genes *CML37*, *CML38* and *CML39*. Plant Mol Biol. 2007;64:683–97. doi:10.1007/s11103-007-9189-0.
28. Sun Q, Huang R, Zhu H, Sun Y, Guo Z. A novel Medicago truncatula calmodulin-like protein (*MtCML42)* regulates cold tolerance and flowering time. Plant J. 2021;108:1069–82. doi:10.1111/tpj.v108.4.
29. Ding H, Qian Y, Fang Y, Ji Y, Sheng J, Ge C. Characteristics of *SlCML39*, a tomato calmodulin-like gene, and its negative role in high temperature tolerance of arabidopsis thaliana during germination and seedling growth. Int J Mol Sci. 2021;22:11479. doi:10.3390/ijms222111479.
30. Munir S, Liu H, Xing Y, Hussain S, Ouyang B, Zhang Y, et al. Overexpression of calmodulin-like (*ShCML44*) stress-responsive gene from Solanum habrochaites enhances tolerance to multiple abiotic stresses. Sci Rep. 2016;6:317.

31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25:402–8. doi:10.1006/meth.2001.1262.
32. Hashim FA, Mabrouk MS, Al-Atabany W. Review of different sequence motif finding algorithms. Avicenna J Med Biotechnol. 2019;11:130–48.
33. Willson J. Exons as enhancers. Nat Rev Genet. 2020;21:68–9. doi:10.1038/s41576-019-0207-2.
34. Shaul O. How introns enhance gene expression. Int J Biochem Cell Biol. 2017;9:145–55.
35. Ding XC, Zhang LP, Hao YW, Xiao SL, Wu ZX, Weixin C, et al. Genome-wide identification and expression analyses of the calmodulin and calmodulin-like proteins reveal their involvement in stress response and fruitripening in papaya. Postharvest Biol Technol. 2018;143:3–27.
36. Nie S, Zhang M, Zhang L. Genome-wide identification and expression analysis of calmodulin-like (CML) genes in Chinese cabbage (*Brassica rapa* L. ssp. pekinensis). BMC Genom. 2017;18(1):842. doi:10.1186/s12864-017-4240-2.
37. Zhao H, Gao Y, Du Y, Du J, Han Y. Genome-wide analysis of the CML gene family and its response to melatonin in common bean (*Phaseolus vulgaris* L.). Sci Rep. 2023;13:1196. doi:10.1038/s41598-023-28445-y.
38. Mohanta TK, Kumar P, Bae H. Genomics and evolutionary aspect of calcium signaling event in calmodulin and calmodulin-like proteins in plants. BMC Plant Biol. 2017;17:38. doi:10.1186/s12870-017-0989-3.
39. Shi J, Du X. Identification, characterization and expression analysis of calmodulin and calmodulin-like proteins in *Solanum pennellii*. Sci Rep. 2020;10:7474. doi:10.1038/s41598-020-64178-y.
40. Clark JW. Genome evolution in plants and the origins of innovation. New Phytol. 2023;240:2204–9. doi:10.1111/nph.19242.
41. Tsai YC, Delk NA, Chowdhury NI, Braam J. Arabidopsis potential calcium sensors regulate nitric oxide levels and the transition to flowering. Plant Signal Behav. 2007;2:446–54. doi:10.4161/psb.2.6.4695.
42. Mithra SVA, Kulkarni K, Srinivasan R. Plant promoters: characterization and applications in transgenic technology. In: Abdin M, Kiran U, Kamaluddin A, editors. Plant biotechnology: principles and applications. Singapore: Springer; 2017. p. 117–72.
43. Xu C, Wang Y, Yang H, Tang Y, Liu B, Hu X, et al. Cold acclimation alleviates photosynthetic inhibition and oxidative damage induced by cold stress in citrus seedlings. Plant Signal Behav. 2023;18(1):2285169. doi:10.1080/15592324.2023.2285169.
44. Xu J, Chen Z, Wang F, Jia W, Xu Z. Combined transcriptomic and metabolomic analyses uncover rearranged gene expression and metabolite metabolism in tobacco during cold acclimation. Sci Rep. 2020;10:5242. doi:10.1038/s41598-020-62111-x.