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ORIGINAL ARTICLE

Genome-wide Identification and Expression Analysis of *CaM/CML* Gene Family in Sacred Lotus (*Nelumbo nucifera*)

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Abstract

Calmodulin (CaM) and calmodulin-like (CML) proteins, a group of Ca^{2+} sensors, play an important role in a large number of different biological processes, including plant growth and development, as well as the biotic and abiotic stress responses. However, *CaM/CML* genes have not been identified in sacred lotus (*Nelumbo nucifera*), an important horticultural plant, and the expressional patterns of these genes are yet to be elucidated. In this study, thirty-four *CaM/CML* genes from *Nelumbo nucifera* were identified. Phylogenetic analysis showed that they could be divided into nine groups. Gene structure and conserved motif analyses demonstrated the conservation and divergence of *CaMs/CMLs* in *Nelumbo nucifera*. Cis-acting elements analysis indicated that they might be related to plant growth and development, abiotic stress, and plant hormones. In addition, expression analysis showed that *NNU-CaMs/CMLs* were differentially expressed in various tissues and responded to calcium treatments in roots. Moreover, weighted gene co-expression network analysis of public transcriptome data of lotus wild variety "China Antique" with different tissues presented the expression connectivity of *NNU-CaMs/CMLs*, which were divided into 11 modules. Gene ontology analysis of the genes in each module demonstrated that *NNU-CaMs/CMLs* may be involved in extensive biological processes, such as synthesis and processing of DNA and RNA, and protein post-translational modification, and each module specifically correlated with the phenotypes including the development of leaves' petiole and lotus seed, implying the wide regulation of *NNU-CaMs/CMLs* in lotus. Taken together, our results will enhance the understanding and lay a foundation for further study of the functions of the *NNU-CaMs/CMLs*.

Keywords Calmodulin · Calmodulin-like · Gene structure · Expression analysis · *N. nucifera*

Introduction

Calcium is an important nutrient, and its ion (Ca^{2+}) is a versatile intracellular messenger in all eukaryotes. In plants, Ca^{2+} acts as a secondary messenger involved in plant growth and development including stress responses. Stimuli from the surrounding environment and developmental processes elicit calcium signals, and the free Ca^{2+} concentration rapidly increases in the cytosol (Sarwat et al. 2013). To maintain Ca^{2+} homeostasis, plant cells have developed calcium channels and pumps for compartmentalization and extrusion of the extra ion (Luan and Wang 2021). Meanwhile, Ca^{2+}-binding proteins with EF-hand motifs that are Ca^{2+}-binding structural motifs bind Ca^{2+} to reduce its levels in the cell. Calmodulin (CaMs) and calmodulin-like proteins (CMLs) (Zielinski 1998), calcium-dependent protein kinase (CDPKs) (Harmon et al. 2000), and calcineurin B-like proteins (CBLs) (Luan et al. 2002) are the three important EF-hand family proteins in plants.

CaM and *CML* gene family had been identified and analyzed in various plant species, including *Arabidopsis* (McCormack and Braam 2003), rice (Boonburapong and Buaboocha 2007), apple (Li et al. 2019), *Brassica napus* (He et al. 2020), Papaya (Ding et al. 2018), Chinese cabbage

Key Message
- A total of 34 *CaM/CML* genes from lotus (*Nelumbo nucifera*) were identified.
- *NNU-CaMs/CMLs* were differentially expressed in various tissues.
- *NNU-CaMs/CMLs* responded to calcium signaling under calcium treatments in roots.
- *NNU-CaMs/CMLs* are involved in extensive biological processes.

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(Nie et al. 2017), woodland strawberry (Zhang et al. 2016), Grapevine (Vandelle et al. 2018), *Lotus japonicas* (Liao et al. 2017), wild tomato (Shi and Du 2020), and tomato (Munir et al. 2016). Genome-wide identification of *CaM/CML* family from plants showed plants encoded more *CMLs* than *CaMs*, and the number is independent of genome size. CaMs are highly conserved proteins containing four EF-hand motifs without any other functional domains. CMLs contain 1 to 6 Ca^{2+}-binding EF-hand motifs (DeFalco et al. 2009). Furthermore, the majority of *CML*s are intron-less, whereas *CaMs* are intron-rich (Mohanta et al. 2017). However, *CaMs/CMLs* have not been identified in *Nelumbo nucifera*.

As Ca^{2+} sensors, *CaMs/CMLs* have been reported to be involved in various developmental processes (Perochon et al. 2011; Campos et al. 2018). In pollen germination and pollen tube elongation in *Arabidopsis thaliana*, the level of pollen germination in loss-of-function mutant *cam2* was reduced (Landoni et al. 2010); AtCML25 regulated the K^+ influx, and loss-of-function mutation in the *AtCML25* caused a major reduction in the rate of pollen germination and the elongation of the pollen tube (Wang et al. 2015b). Mutation of *cml*24 leads to an obvious reduction of root length and decreased lateral root density in *Arabidopsis* (Zha et al. 2016). Cotton *GhCaM7* promoted the elongation of cotton fiber by regulating the production of reactive oxygen species (ROS) (Tang et al. 2014). AtCML39 promoted the light-dependent seedling establishment and loss-of-function *cml39* mutants displayed growth arrest after germination in the absence of exogenous sucrose (Bender et al. 2013). In addition, AtCML39 was involved in regulating ovule and fruit development where the loss of *AtCML39* resulted in reduced seed number in shorter siliques and the number of ovules (Midhat et al. 2018).

In addition, the roles of *CaMs/CMLs* in plant responses to both abiotic and biotic stimuli have also been reported (Ranty et al. 2016; Zeng et al. 2015). *AtCML20* acts as a negative regulator of ABA and drought stress responses in *Arabidopsis* (Wu et al. 2017), while *AtCML37* is a positive regulator of ABA accumulation induced by drought stress (Scholz et al. 2015). Besides, *AtCML37* functions as an active regulator of plant resistance to lepidopteran herbivores (Scholz et al. 2014). Overexpression of *AtCML8* enhances the plants' resistance to pathogenic bacteria (Zhu et al. 2017), indicating that *AtCML8* is a positive regulatory factor in plant immunity. Overexpression of the *GmCaM4* gene in soybean enhanced its resistance to three plant pathogens and improved its tolerance to high salt conditions (Rao et al. 2014). Moreover, *AtCML41*, *AtCML46*, and *AtCML47 in Arabidopsis* and type III *CaM* subtype in Tobacco had been documented to participate in the biotic and abiotic stress response (Xu et al. 2017; Lu et al. 2018; Takabatake et al. 2007).

Nelumbo nucifera (*N. nucifera*) belongs to the small family of *Nelumbonaceae* (Wang and Zhang 2005), and is named as sacred lotus due to its significance in the religions of Buddhism and Hinduism (Shen-Miller 2002). It is also a popular ornamental, vegetable, and medicinal plant with great economic value (Lin et al. 2019). It is critical to obtain a deeper understanding on its growth and development regulation, as well as its response to different stimuli. Since Ca^{2+} is one of the most important secondary messengers, it is necessary to figure out its signaling. Nevertheless, there is limited information about *CaMs/CMLs* and their functions in lotus. In this study, we have identified 34 *CaMs/CMLs* in *N. nucifera* based on the genome-wide analysis and have performed bioinformatics analysis that included phylogenetic analysis, gene structures, conserved motifs, and promoter cis-acting elements. The expression patterns in 15 different tissues and weighted gene co-expression network analysis were investigated. Additionally, *CaMs/CMLs* in response to calcium signaling under calcium treatment were studied. The findings of this study provide potential clues in illuminating the detailed function of *CaMs/CMLs* involved in the growth, development, and calcium signaling response of *N. nucifera* in the future.

Materials and Methods

Plant Materials and Treatments

The seeds of China Antique (CA, wild lotus) were placed in the tap-water and germinated in the growth chamber maintained at 22–28 °C, with a photoperiod of 12 h light/12 h dark. For calcium treatment, 2-week-old sacred lotus seedlings were transferred to 10 mM $CaCl_2$, 10 mM EDTA, and H_2O (control) until collected for further experiments. Roots were collected at 1, 2, 4, and 12 h after $CaCl_2$, EDTA, and H_2O treatments. Three biological replicates were set for each treatment. All the samples were frozen in liquid nitrogen immediately and stored at − 80 °C until used for RNA extraction.

Identification of *CaM/CML* Family Genes of Lotus

The protein sequences of CaM/CML in *Arabidopsis* (AtCaM/AtCML) (McCormack and Braam 2003) and rice (OsCaM/OsCML) (Boonburapong and Buaboocha 2007) were downloaded from TAIR database (https://www.arabidopsis.org/) and Rice Annotation Project Database (https://rapdb.dna.affrc.go.jp/index.html), respectively. The lotus genomic sequences and protein sequences were downloaded from the lotus database (http://lotus-db.wbgcas.cn/) (Ming et al. 2013; Wang et al. 2015a). All the AtCaM/CML and OsCaM/CML protein sequences were used to search for lotus CaM and CML

proteins by local BALSTP in BioEdit version 7.0.9.0 with an e-value lower than 10^{-5}. To prove the reliability of the candidate sequences, the core domain of the sequences was analyzed by Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/smart/batch.pl). Proteins containing the EF-hand domain as well as not any other identifiable domains were considered as putative *N. nucifera* CaM/CML proteins (NNU-CaMs/CMLs) (Wang et al. 2015a). The theoretical molecular weight (Mw) and isoelectric point (pI) were predicted using an online pI/Mw tool (https://web.expasy.org/compute_pi/). The subcellular location was predicted in the online software WoLF PSORT (https://wolfpsort.hgc.jp/).

Gene Structure, Conserved motif, and Promoter Analysis

The intron–exon gene structure of *NNU-CaMs/CMLs* was analyzed by alignment of coding sequences (CDS) and its corresponding genomic DNA sequences in GSDS 2.0 (http://gsds.gao-lab.org/) (Hu et al. 2015). MEME (http://meme-suite.org/tools/meme) (Bailey et al. 2009) was used to determine the conserved motif of NNU- CaMs/CMLs, and the number of motifs was set to 10, which were further used in the InterPro database (https://www.ebi.ac.uk/interpro/search/sequence/) to annotate motif. The cis-acting elements of 2 kb upstream promoter sequences of *NNU-CaMs/CMLs* were analyzed by the online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Phylogenic Analysis

Multiple sequences of the identified CaMs/CMLs from *Arabidopsis* (McCormack and Braam 2003), rice (Boonburapong and Buaboocha 2007), and *N. nucifera* were aligned by ClustalW. The phylogenetic tree was constructed by Neighbor-Joining using MEGA-X (Kumar et al. 2018) with the Bootstrap method, and the number of Bootstrap Replications was set at 1000. Finally, beautification of the phylogenetic tree was done using the online tool ITOL (https://itol.embl.de/) (Letunic and Bork 2016).

Expression Profiling of *CaM/CML* Family Genes

The genome-wide transcriptome data of sacred lotus from 15 different tissues or developmental stages including anther, carpel, cotyledon, seed coat, and rhizome (Zhang et al. 2019; Li et al. 2018, 2020; Ming et al. 2013) were downloaded from National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), which were accessed and filtered through FastQC (V0.11.3) and Trimmomatic (V0.38) (Bolger et al. 2014). The obtained clean reads were aligned to the reference genome of *N.nucifera* from lotus database (http://lotus-db.wbgcas.cn/) (Wang et al. 2015a) using Hisat2 (v2.0.5) (Kim et al. 2015), and the featureCount (V1.6.4) (Liao et al. 2014) was applied to quantify and standardize the read count and TPM (transcripts per kilobase per million) value of each expressed gene. The mRNA expression data of *NNU-CaMs/CMLs* were extracted from genome-wide expression data, and then, the heatmap was drawn via the R package "pheatmap" (www.r-project.org). The weighted gene co-expression network analysis (WGCNA) method (Langfelder and Horvath 2008) was utilized to further characterize the expression profile of *CaM/CML* family genes in *N.nucifera*. All of the genes were used for constructing an expression matrix, and genes with similar expression patterns were clustered into the same module. The relationships between the genes in the module and the different samples were calculated, and the modules containing *CaM/CML* family genes were identified. In each module, the co-expressed genes associated with *CaM/CML* family genes were applied to analyze the gene ontology (GO) using the online software AgriGO V2.O (http://systemsbiology.cau.edu.cn/agriGOv2/index.php) (Du et al. 2010).

Gene Expression Analysis by Quantitative Real-Time PCR

The TRIZOL reagent was used to extract total RNA, and the kit of HiScript II Q RT SuperMix for qPCR with gDNA wiper (Vazyme, China) was applied to synthesize cDNA according to manufacturer's protocol. The gene-specific primers were designed through an online software Primer-3Plus (https://primer3plus.com/). qRT-PCR was conducted using gene-specific primers (Table S1) in a 10μL reaction with a 2 × iTaq™ Universal SYBR Green Supermix (Bio-Rad, USA) under the annealing temperature of 54 °C for 10 s, in which the internal reference NnActin (NNU-02553) (Deng et al. 2015) was used to normalize the expression data. Relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ (cycle threshold) method (Livak and Schmittgen 2001).

Results

Identification and Phylogenetic Analysis of *CaM/CML* Genes in Lotus

In order to identify the *NNU-CaMs/CMLs* gene family, 56 CaMs/CMLs in *Arabidopsis* and 37 CaMs/CMLs in rice (Table S2) were blasted against the lotus protein sequences using local BLASTP in BioEdit. A total of 217 putative lotus protein sequences were identified by local BLASTP under the given criterion, which was further subjected to analysis in Pfam and SMART. Pfam and SMART predicted the presence of EF-hand motif in 82 and 34 protein sequences,

respectively. A total of 34 lotus proteins (Table S3) predicted by both were finally selected as the presumed *NNU-CaM/CML* family proteins. Among all the NNU-*CaM/CML* proteins, 10, 7, and 4 had four, three, and two EF-hand motifs (Table 1). The numbers of EF-hand motifs in the remaining 13 proteins were inconsistently predicted by the two software. The length of the protein sequences ranged from 82 amino acids (NNU_17378) to 294 amino acids

Table 1 The characteristics of 34 *NNU-CaMs/CMLs*

ID	CDS length	Amion acid	pI[a]	MW[b]	No. of EF-hand[c]		Chromosolar location	Subcellular localization[d]
					Pfam	SMART		
NNU_02678	438	145	4.09	16.4	3	3	scaffold_1	extr: 5, chlo: 4, cyto: 3, nucl: 2
NNU_09864	576	191	4.83	21.6	2	3	scaffold_1	nucl: 8.5, cyto_nucl: 5, chlo: 2, mito: 2, plas: 1
NNU_18361	483	160	3.96	17.3	3	3	scaffold_1	chlo: 4, cyto: 4, nucl: 2, extr: 2, cysk: 2
NNU_11273	480	159	4.52	17.9	2	3	scaffold_1	extr: 4, chlo: 3, nucl: 3, cyto: 3, golg: 1
NNU_26454	684	227	4.54	25.2	4	4	scaffold_2	extr: 6, chlo: 4, vacu: 2, cyto: 1, mito: 1
NNU_26426	627	208	4.51	23.4	4	4	scaffold_2	chlo: 7, mito: 4, nucl: 3
NNU_09004	492	163	4.39	18.1	2	4	scaffold_2	nucl: 5.5, nucl_plas: 4.5, cyto: 4, plas: 2.5, mito: 1,
NNU_26510	621	206	4.80	23.7	1	2	scaffold_2	chlo: 6, plas: 2.5, nucl: 2, mito: 2, golg_plas: 2,
NNU_23744	702	233	4.53	25.8	4	4	scaffold_3	chlo: 12, extr: 2
NNU_09260	621	206	4.35	22.8	3	3	scaffold_3	chlo: 10, nucl: 2, mito: 2
NNU_17382	495	164	4.89	18.3	4	4	scaffold_4	chlo: 6, mito: 5, nucl: 1, cyto: 1, extr: 1
NNU_17533	450	149	4.16	17.0	4	4	scaffold_4	nucl: 6, cyto: 2, plas: 2, extr: 2, chlo: 1, cysk: 1
NNU_02112	426	141	4.35	16.1	2	4	scaffold_4	cyto: 6, chlo: 2, mito: 2, extr: 2, nucl: 1, cysk: 1
NNU_07289	591	196	4.99	22.4	2	2	scaffold_4	chlo: 8, nucl: 3, extr: 2, golg: 1
NNU_17378	249	82	4.31	9.2	2	2	scaffold_4	mito: 7, nucl: 4.5, cysk_nucl: 3, cyto: 1, extr: 1
NNU_03952	492	163	4.41	18.1	4	4	scaffold_5	nucl: 7, chlo: 3, extr: 2, plas: 1, golg: 1
NNU_20720	573	190	4.90	21.0	4	4	scaffold_5	nucl: 8, mito: 3, chlo: 2, golg_plas: 1
NNU_22133	432	143	4.32	16.3	2	4	scaffold_5	nucl: 7, chlo: 4, cyto: 2, extr: 1
NNU_00630	486	161	4.34	17.8	3	4	scaffold_5	cyto: 6, nucl: 3.5, nucl_plas: 3, mito: 2, plas: 1.5
NNU_07897	444	147	4.25	16.7	2	3	scaffold_6	cyto: 7, nucl: 4, extr: 2, cysk: 1
NNU_07895	459	152	4.19	17.5	3	3	scaffold_6	chlo: 5, cyto: 4, nucl: 3, plas: 1, extr: 1
NNU_05464	633	210	4.55	23.5	3	3	scaffold_6	nucl: 8, mito: 3, chlo: 2, golg_plas: 1
NNU_10822	450	149	4.11	16.8	4	4	scaffold_7	nucl: 5, mito: 3, extr: 3, cyto: 2, chlo: 1
NNU_24913	612	203	4.86	22.9	4	4	scaffold_8	chlo: 8, nucl: 3, mito: 3
NNU_15888	492	163	4.45	18.2	4	4	scaffold_8	nucl: 7, nucl_plas: 6, plas: 3, cyto: 2, mito: 1, cysk: 1
NNU_06974	642	213	4.87	24.6	2	3	scaffold_8	chlo: 6, nucl: 4, mito: 3, cyto: 1
NNU_13842	489	162	4.32	17.7	2	4	scaffold_9	cyto: 5, extr: 4, chlo: 2, nucl: 2, golg: 1
NNU_12734	555	184	4.34	20.7	1	2	scaffold_10	cyto: 5, chlo: 3, nucl: 2, pero: 2, extr: 1, golg: 1
NNU_19002	684	227	6.09	25.9	3	3	scaffold_11	nucl: 10, cyto_nucl: 6.3, nucl_plas: 5.8, cyto: 1.5,
NNU_09549	582	193	4.91	21.6	2	4	scaffold_14	nucl: 13, cyto: 1
NNU_16648	591	196	4.68	22.1	3	3	scaffold_15	cyto: 6, nucl: 3.5, nucl_plas: 2.5, extr: 2, mito: 1
NNU_14160	660	219	4.76	23.9	3	4	scaffold_19	mito: 7, nucl: 4, chlo: 2, golg_plas: 1
NNU_22775	837	278	6.42	30.0	2	2	scaffold_21	extr: 4, chlo: 3, plas: 3, vacu: 2, nucl: 1, E.R.: 1
NNU_22784	885	294	6.89	31.6	2	2	scaffold_21	plas: 4.5, chlo: 4, E.R._plas: 3, mito: 2, cyto: 1, extr: 1,

[a]pI, theoretical isoelectric point

[b]MW, theoretical molecular weight, kDa

[c]No. of EF-hand, number of EF hands based on the prediction by Pfam and SMART

[d]The prediction of subcellular location in WoLF PSORT (https://wolfpsort.hgc.jp/). extr, extracellular; chlo, chloroplast; cyto, cytosol; nucl, nuclear; cyto_nucl, nuclear and cytoplasmic; mito, mitochondria; plas, plasma membrane; cysk, cytoskeleton; golg, golgi; vacu, vacuolar; membrane; nucl_plas, nuclear and plasma membrane; golg_plas, golgi and plasma membrane; pero, peroxisome; E.R., endoplasmic reticulum

(NNU_22784). The theoretical molecular weights (MW) and isoelectric point (pI) ranged from 9.2 to 31.6 kDa and from 3.96 to 6.89, respectively. The genome of the sacred lotus has been assembled into nine major anchored megascaffolds and several small scaffolds (Ming et al. 2013). Among all the *NNU-CaM/CML* genes, 27 were distributed on 9 of the largest scaffolds (Table 1). The prediction results of subcellular localization indicated that most of the NNU-CaMs/CMLs were located on the nuclear and cytosol, but some were plastid proteins such as chloroplast proteins and mitochondria proteins. Moreover, an NNU-CaM/CML was predicted to be plasma membrane protein (Table 1).

The phylogenetic tree was constructed by Neighbor-Joining and Bootstrap method to study the evolutionary relationship of *CaM/CML* family genes in *Arabidopsis*, rice, and lotus (Fig. 1). The results showed that all *CaM/CML* family genes were divided into 12 groups (Group 1 to Group 12), and 34 *NNU-CaMs/CMLs* were distributed in 9 groups including Group 1, Group 2, and Group 6 to 12. Groups 8 and 10 were the largest group that contained 7 members of the *NNU-CaM/CML* genes, respectively. Groups 1 and 2 only contained one *NNU-CaM/CML* gene.

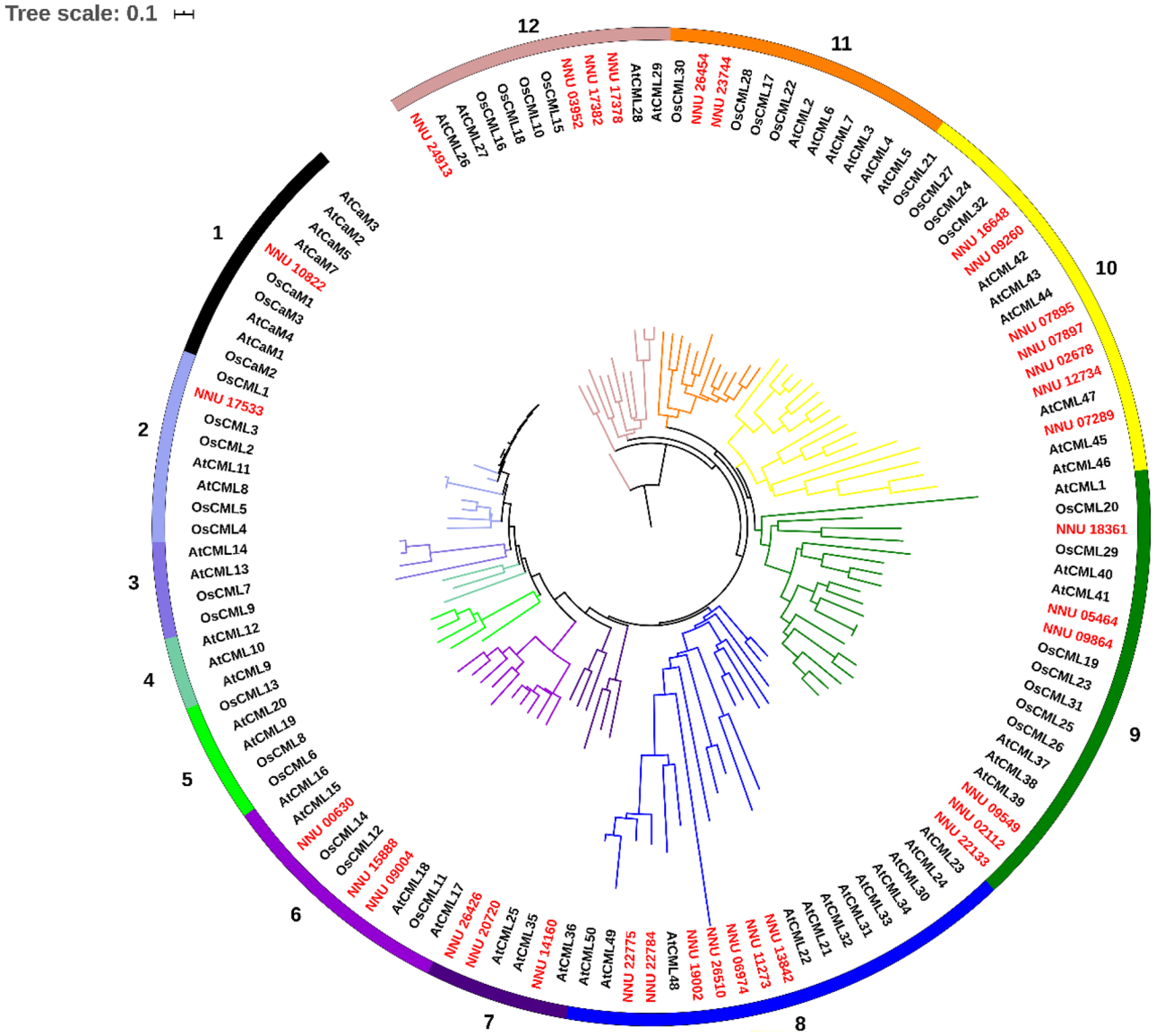


Fig. 1 Phylogenetic analysis of CaM/CML proteins from *Arabidopsis*, rice, and lotus. The numbers 1–12 indicate different groups, and different groups are represented by different colors in the outermost circle, and the color on the phylogenetic tree is the same as the group color. *NNU-CaMs/CMLs* are distinguished using red color. The unrooted neighbor-joining (Nj) phylogenetic tree was constructed by MEGA-X. Protein sequences were downloaded from TAIR database (https://www.arabidopsis.org/), Rice Annotation Project Database (https://rapdb.dna.affrc.go.jp/index.html), and the lotus database (http://lotus-db.wbgcas.cn/) (Wang et al. 2015a), respectively

Gene Structure, Conserved Motifs, and Analysis of the Promoters Cis-Acting Elements

Exon–intron structure analysis showed that seven *NNU-CaM/CML* genes contained one to seven introns and the remaining 27 *NNU-CaM/CML* genes had only one exon, in which NNU_10822 contained only one intron, while NNU_06974 contained seven introns (Fig. 2). A total of 10 conserved motifs were identified in 34 NNU-CaM/CML proteins using MEME. Members of NNU-CaMs/CMLs contained different number of motifs, and each NNU-CaM/CML protein had two to six conserved motifs. A total of 18 NNU-CaM/CML proteins contained four conserved motifs. Motif sequence analysis showed that motif 1–7 were EF-hand domains for Ca^{2+} binding and all the NNU-CaM/CML proteins had Motif 1 and motif 2 (Fig. 3). A total of 887 cis-acting elements were discovered in *NNU-CaM/CML* genes, including 430 light-responsive elements and 244 hormone-responsive elements (Fig. 4). In addition, numerous cis-acting elements related to plant growth and development, abiotic stress, and plant hormones were found in the promoter region of *NNU-CaM/CML* genes, including two development-related elements CAT-box and GCN4_motif; eight hormone-responsive elements such as ABRE, CGTCA-motif, AuxRR-core, TCA-element, and TATC-box; five abiotic stress–related elements such as MBS, ARE, and LTR; and fifteen light-responsive elements.

Fig. 3 Motifs analysis of 34 *N. nucifera* CaMs/CMLs. Ten motifs identified by the MEME tool are represented by different colored boxes, and their sequences are shown at the bottom. The motifs of NNU-CaMs/CML proteins were grouped according to the phylogenetic classification in Fig. 1

Expression Characterization of *NNU-CaM/CML* Genes

To clarify the potential function of *NNU-CaMs/CMLs*, their expression patterns in 15 different tissues including

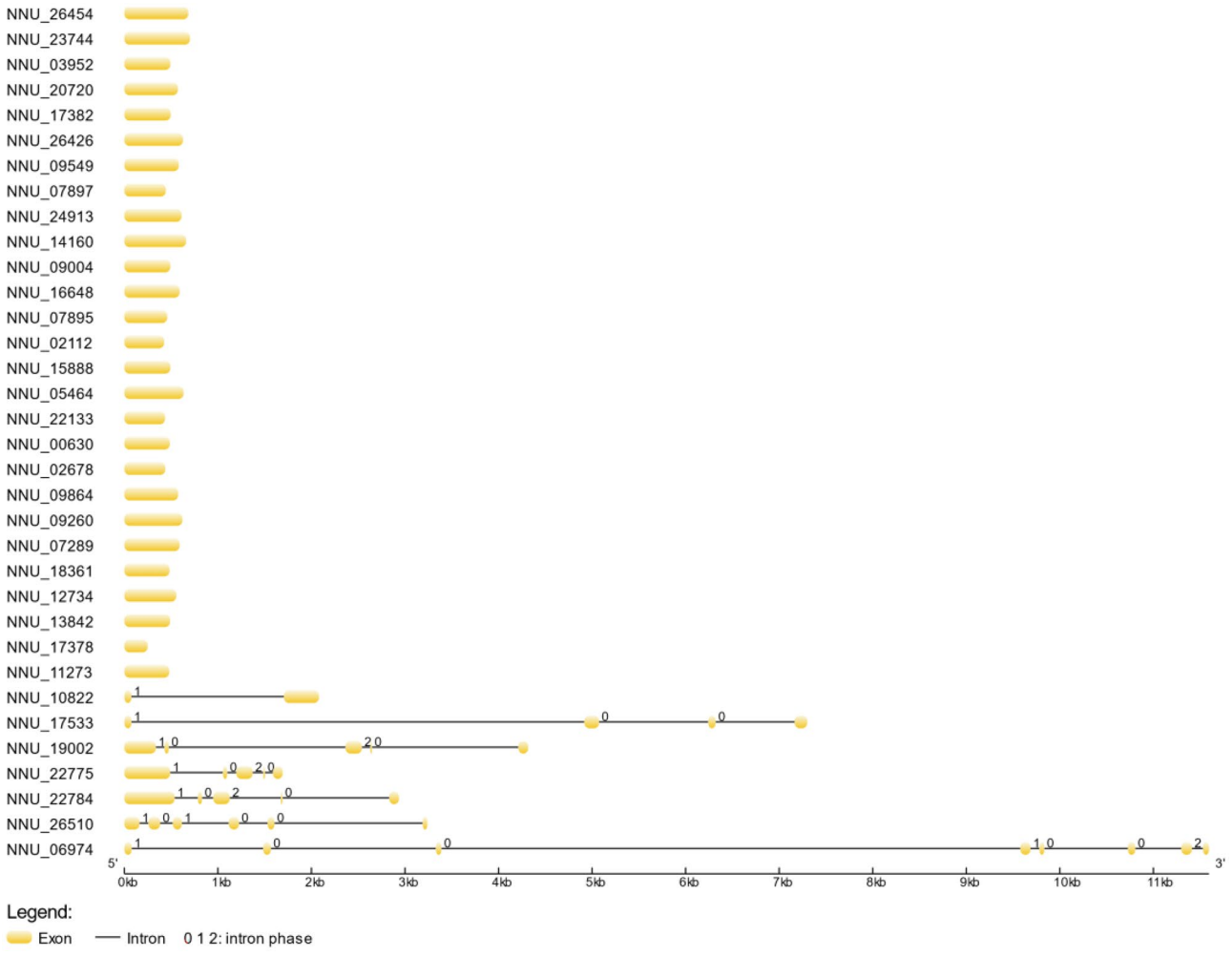
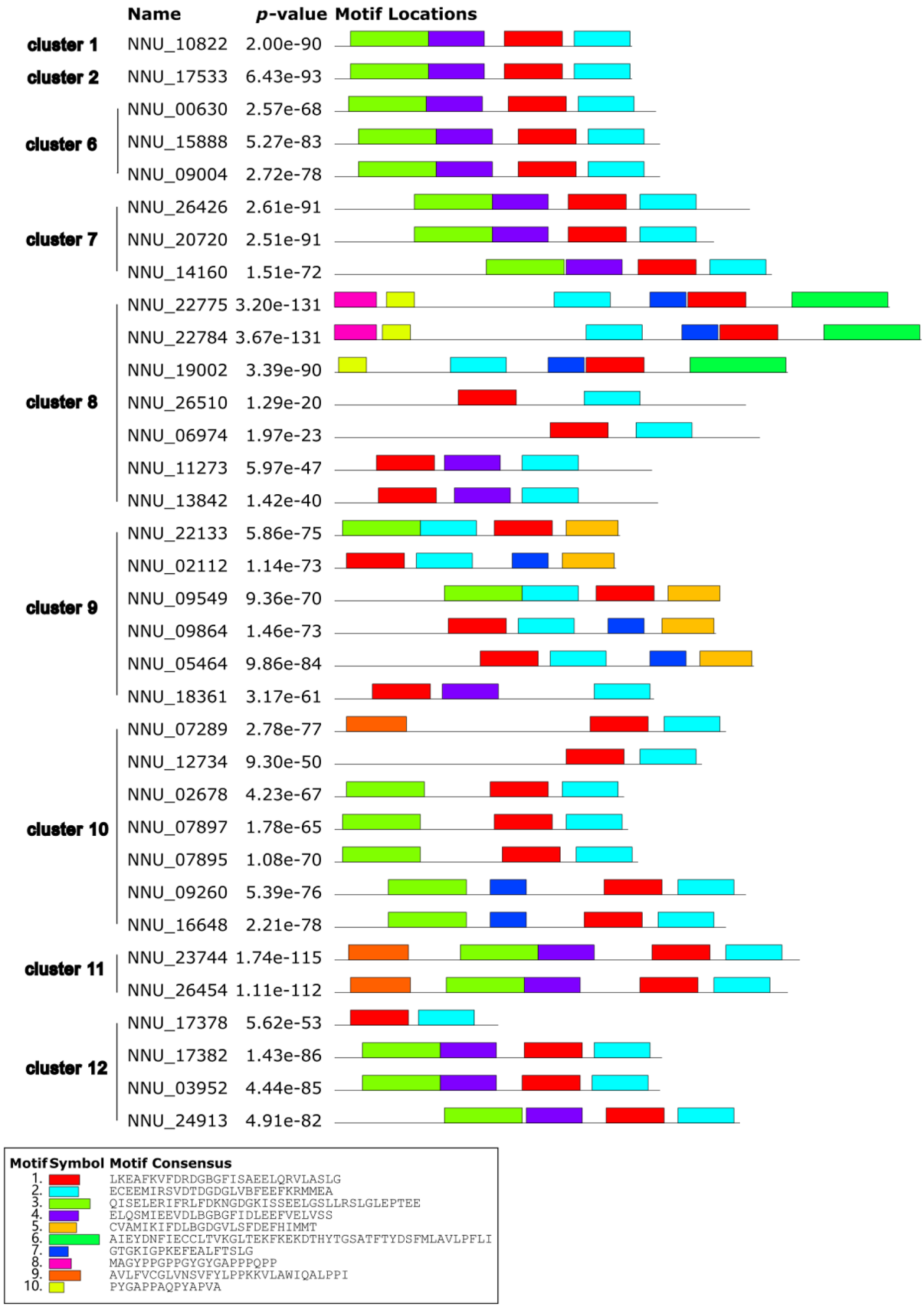


Fig. 2 Gene structure analysis of 34 *N. nucifera CaMs/CMLs*. Exons and introns arrangement of *NNU-CaM/CML* genes are indicated by yellow shapes and black lines, respectively. The length of each *NNU-CaMs/CMLs* can be estimated using the scale at the bottom


Name
p-value
Motif Locations
cluster 1
NNU_10822 2.00e-90
cluster 2
NNU_17533 6.43e-93
cluster 6
NNU_00630 2.57e-68
NNU_15888 5.27e-83
NNU_09004 2.72e-78
cluster 7
NNU_26426 2.61e-91
NNU_20720 2.51e-91
NNU_14160 1.51e-72
cluster 8
NNU_22775 3.20e-131
NNU_22784 3.67e-131
NNU_19002 3.39e-90
NNU_26510 1.29e-20
NNU_06974 1.97e-23
NNU_11273 5.97e-47
NNU_13842 1.42e-40
cluster 9
NNU_22133 5.86e-75
NNU_02112 1.14e-73
NNU_09549 9.36e-70
NNU_09864 1.46e-73
NNU_05464 9.86e-84
NNU_18361 3.17e-61
cluster 10
NNU_07289 2.78e-77
NNU_12734 9.30e-50
NNU_02678 4.23e-67
NNU_07897 1.78e-65
NNU_07895 1.08e-70
NNU_09260 5.39e-76
NNU_16648 2.21e-78
cluster 11
NNU_23744 1.74e-115
NNU_26454 1.11e-112
cluster 12
NNU_17378 5.62e-53
NNU_17382 1.43e-86
NNU_03952 4.44e-85
NNU_24913 4.91e-82
Motif Symbol Motif Consensus
1. LKEAFKVFDRDGBGFISAEELQRVLASLG
2. ECEEMIRSVDTDGDGLVBFEEFKRMMEA
3. QISELERIFRLFDKNGDGKISSEELGSLLRSLGLEPTEE
4. ELQSMIEEVDLBGBGFIDLEEFVELVSS
5. CVAMIKIFDLBGDGVLSFDEFHIMMT
6. AIEYDNFIECCLTVKGLTEKFKEKDTHYTGSATFTYDSFMLAVLPFLI
7. GTGKIGPKEFEALFTSLG
8. MAGYPPGPPGYGYGAPPPQPP
9. AVLFVCGLVNSVFYLPPKKVLAWIQALPPI
10. PYGAPPAQPYAPVA

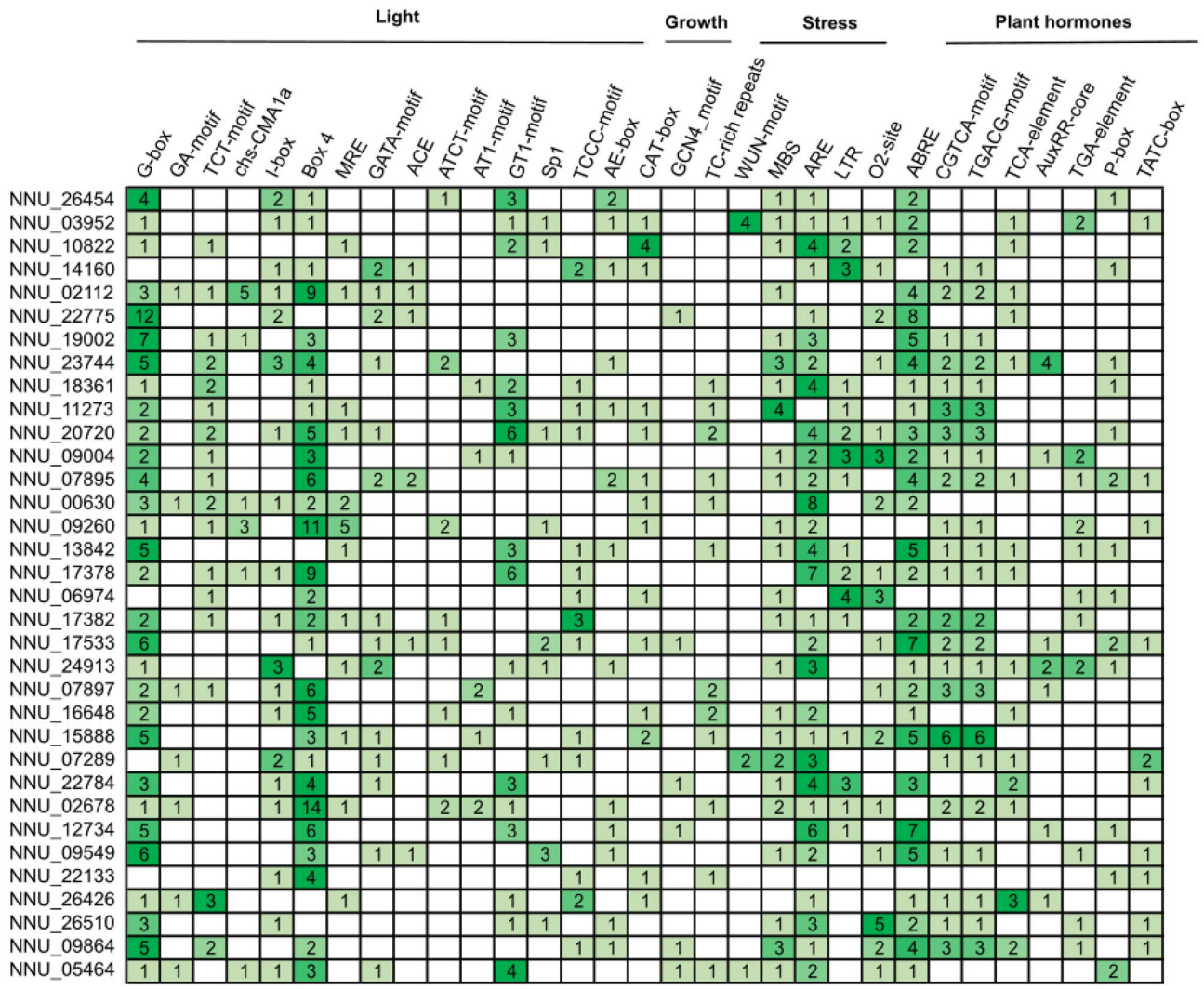


Fig. 4 Cis-acting elements analysis of *N. nucifera CaM/CML* genes' promoters. The numbers indicate the count of cis-acting elements, and the color depth shows the amount. The more the quantity, the darker the color

cotyledon, petiole (initial vertical leaves' petiole and floating leaves' petiole), seed coat (6, 12, and 18 days after pollination), carpel (pollinated and unpollinated), anther (mature and immature), receptacle (mature and immature), petal, rhizome apical meristem, and rhizome elongation zone derived from published transcriptome data (Zhang et al. 2019; Li et al. 2018, 2020; Ming et al. 2013) were analyzed (Fig. 5). All *NNU-CaM/CML* genes had higher expression in the petiole, seed coat (DAP6, DAP12, DAP18), anther (mature and immature), rhizome apical meristem, and rhizome elongation zone, and most of the *NNU-CaM/CML* genes had lower expression in the cotyledon, carpel, receptacle, and petal. NNU_07289, NNU_02678, NNU_12734, NNU_07897, and NNU_16648 had the highest expression in initial floating leaves' petiole floating on the water surface, whereas NNU_20720, NNU_09004, NNU_00630, NNU_26426, NNU_17318, NNU_09260, and NNU_06974 showed higher expression levels in immature-anther compared with other tissues. NNU_26510, NNU_09549, NNU_17382, NNU_26454, and NNU_11273 had low expression in most tissues.

Expression Analysis of *NNU-CaMs/CMLs* in Response to Calcium Signaling

To understand the potential regulation of *NNU-CaM/CML* genes in response to calcium signaling, expression profile of *NNU-CaM/CML* genes under high concentration of calcium and calcium inhibitor (EDTA) treatments was analyzed using qRT-PCR (Fig. 6). Fifteen candidate *NNU-CaM/CML* genes were selected based on the phylogenetic tree classification (Table S1). The expression of fourteen *NNU-CaMs/CMLs*

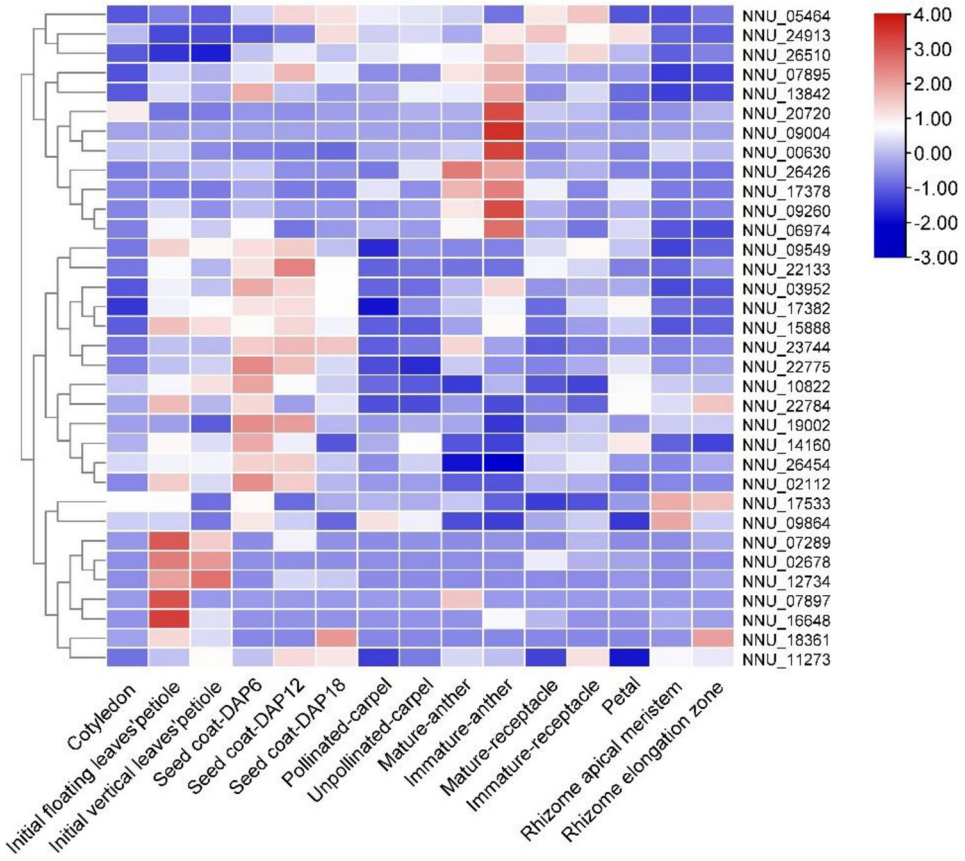


Fig. 5 Expression analysis of *N. nucifera CaM/CML* genes in different tissues. The transcriptome data were obtained from NCBI (Zhang et al. 2019; Li et al. 2018, 2020; Ming et al. 2013). Red and blue represent high and low expression intensity of *NNU-CaM/CML* genes in the heat map, respectively. The phylogenetic tree is shown on the left. The different tissues are noted on the bottom and on the right is *NNU-CaM/CML* gene ID. DAP, days after pollination

was altered in response to calcium and EDTA treatment, and only NNU_15888 had no specific trend after 10 mM $CaCl_2$ and EDTA treatment. Among them, the expression levels of nine genes (NNU_03952, NNU_12734, NNU_09004, NNU_09864, NNU_17533, NNU_11273, NNU_20720, NNU_23744, NNU_22784) increased within 2 h after the calcium treatment. The highest expression levels were observed for NNU_09004 and NNU_09864, which showed 15- and 20-fold higher expression levels at 2 h in 10 mM $CaCl_2$ treatment than control. After 10 mM EDTA treatment, the relative quantitative expression of *NNU-CaMs/CMLs* had no distinct change in the early stage. These results suggest that increased concentration of Ca^{2+} affected the *NNU-CaMs/CMLs* spatial expression patterns and *NNU-CaMs/CMLs* are involved in calcium response.

NNU-CaMs/CMLs Involved in Diverse Biological Processes

In order to elucidate the potential function of *NNU-CaMs/CMLs* involvement in growth and development, the weighted gene co-expression network, GO enrichment of genes co-expressed with *NNU_CaMs/CMLs*, and the relationships between traits and modules involved in *NNU-CaMs/CMLs* were analyzed (Fig. 7). A total of 25 modules were identified based on the co-expression network, and thirty-four *NNU-CaM/CML* genes were divided into 11 different modules including green module, black module, brown module, turquoise module, pink module, yellow module, tan module, blue module, light-cyan module, dark-green module, and green-yellow module (Fig. 7a). The number of *NNU-CaMs/CMLs* in each module ranged from one to eight. Eight *NNU-CaM/CML* genes (NNU_20720, NNU_09004, NNU_07895, NNU_00630, NNU_09260, NNU_13842, NNU_17378, and NNU_06974) were assigned to brown module, which contained the largest number of *NNU-CaM/CML* genes. The second was the green module, which contained 7 *NNU-CaM/CML* genes (NNU_26454, NNU_03952, NNU_10822, NNU_14160, NNU_02112, NNU_22775, and NNU_19002). Only one *NNU-CaM/CML* gene was assigned to the blue (NNU_26426), light-cyan (NNU_26510), dark-green (NNU_09864), and green-yellow (NNU_05464). The number of co-expression genes, which had similar expression patterns with *NNU-CaMs/CMLs*, ranged from 153 (NNU_09864) to 4776 (NNU_17533), and the average number of genes was 1560. The results showed that *NNU-CaMs/CMLs* co-expressed with numerous lotus genes.

GO enrichment analysis of genes in each module connected by *NNU-CaM/CML* genes showed that 9 modules had significant Go terms except tan and green-yellow module

(Fig. 7b). The genes in the turquoise module were involved in a large number of biological processes, mainly related to the synthesis and processing of DNA and RNA. The process of protein post-translational modification was simultaneously enriched in three modules, including the dark-green, green, and pink module. The genes in the light-cyan module participated in protein folding and the genes in the brown module were mainly involved in the biological process of transport. Module-trait relationships analysis displayed a significant correlation between different modules and phenotypes. The nine modules including green, black, pink, yellow, dark-green, turquoise, brown, blue, and light-cyan modules were significantly correlated with different tissues (Fig. 7c). The brown module was significantly correlated with immature anther ($R = 0.99$, $p = 9e-14$), the dark-green module was significantly correlated with rhizome apical meristem ($R = 0.99$, $p = 2e-12$), the development of seed coat was significantly associated with the green module (DAP6, $R = 0.96$, $p = 1e-08$) and the black module (DAP18, $R = 0.95$, $p = 1e-07$), and the petiole types were correlated with pink (floating leaves' petiole, $R = 0.98$, $p = 4e-10$), and yellow module (vertical leaves' petiole, $R = 0.78$, $p = 7e-04$), indicating that different *NNU-CaM/CML* genes may involve in different development processes.

Fig. 7 Weighted gene co-expression network analysis (WGCNA) of *NNU-CaMs/CMLs*. **a** Gene module of co-expression. The number of co-expression genes is indicated by the height of the column, and different colors show different weight ranges. The module of each *NNU-CaMs/CMLs* is located at bottom. **b** GO enrichment. The different modules distinguished using red color and green shape showed the biological process involved. **c** Analysis of the module-trait relationships. Each row represents a module, and each column represents a sample. The numbers on the top and bottom of each cell represent the correlation and significant *p*-values, respectively

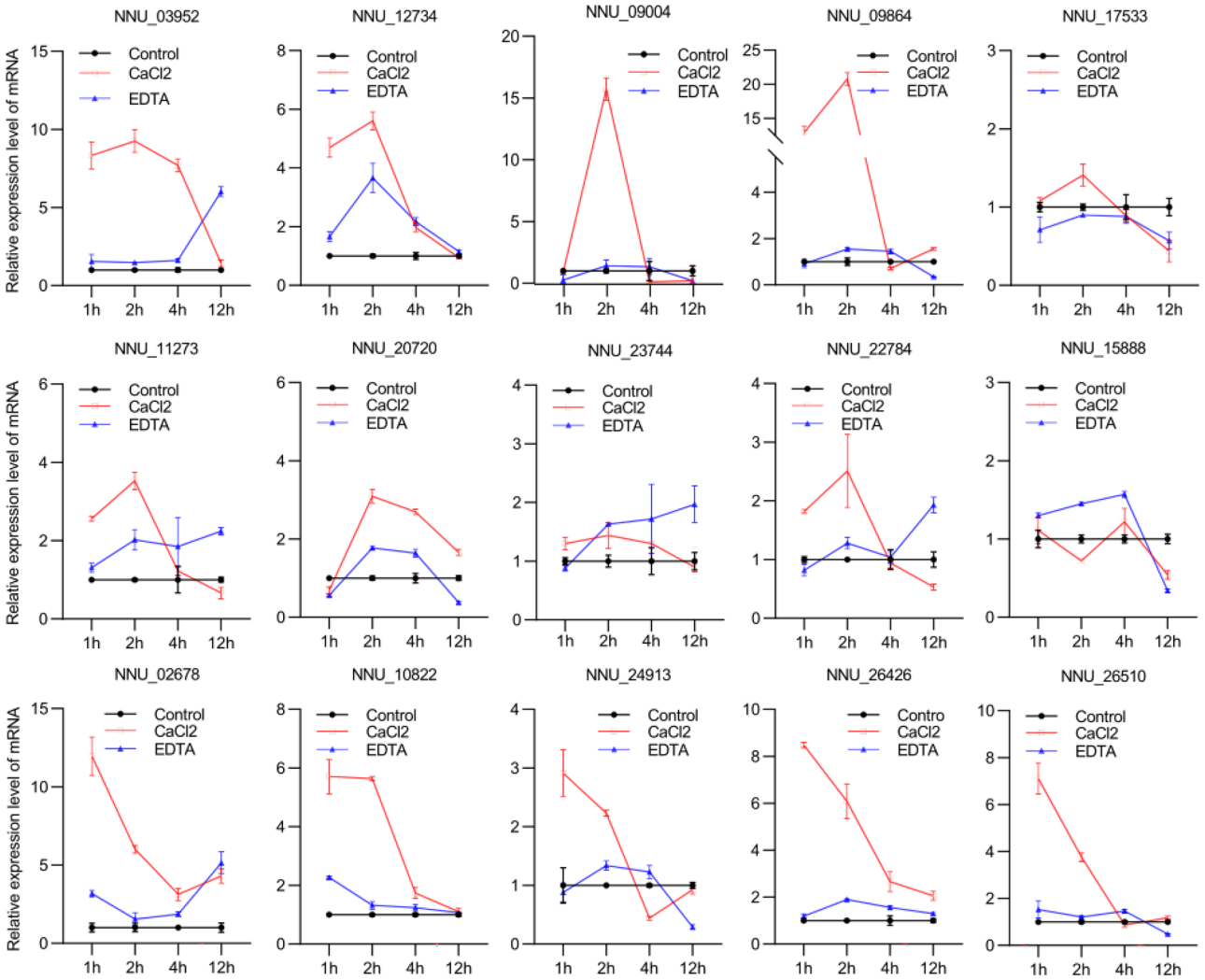
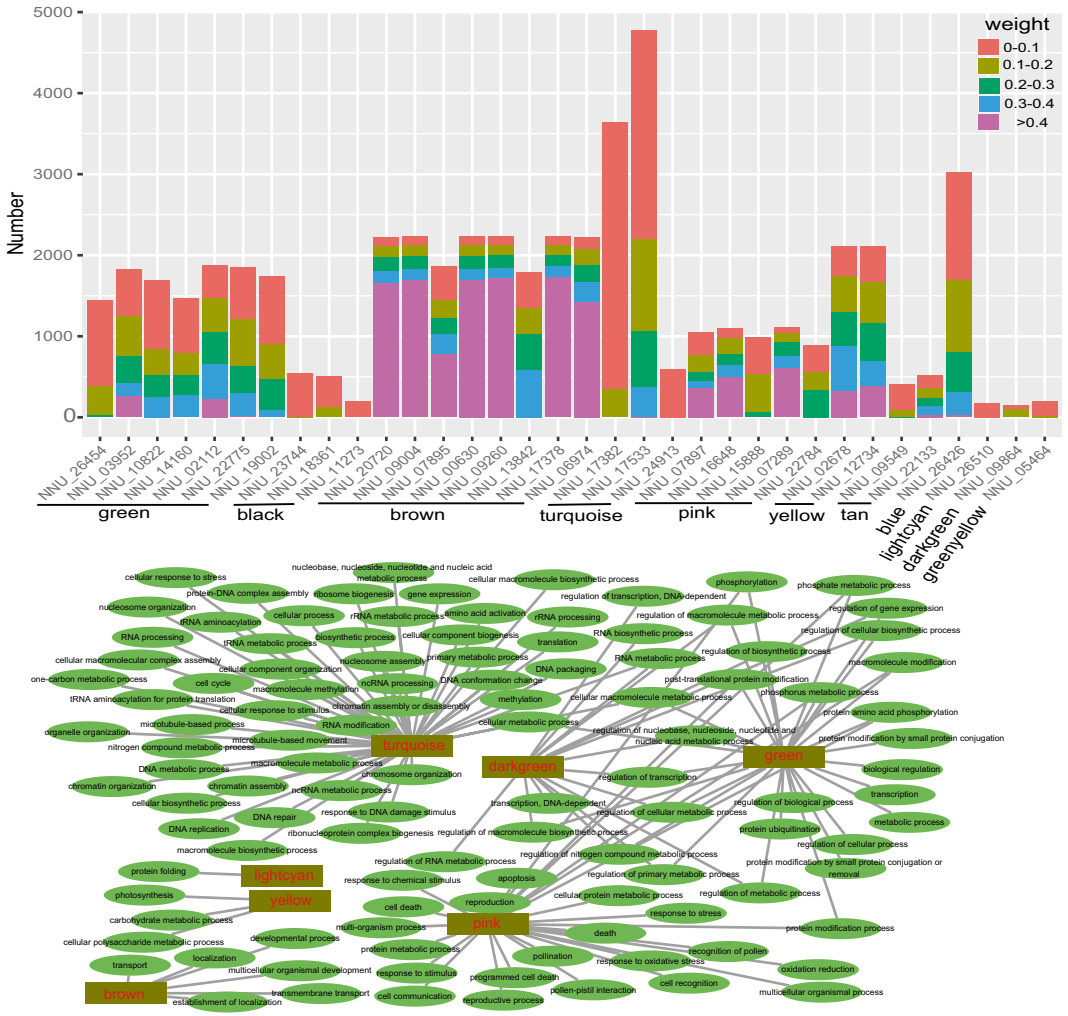


Fig. 6 Expression patterns of *CaM/CML* genes under calcium treatment. The red, blue, and black lines represent 10 mM $CaCl_2$, 10 mM EDTA, and Control (H_2O) treatment. Root were collected at 1, 2, 4, and 12 h after treatment. The error bars represent the standard deviations of three replicates


weight
0-0.1
0.1-0.2
0.2-0.3
0.3-0.4
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Number
5000
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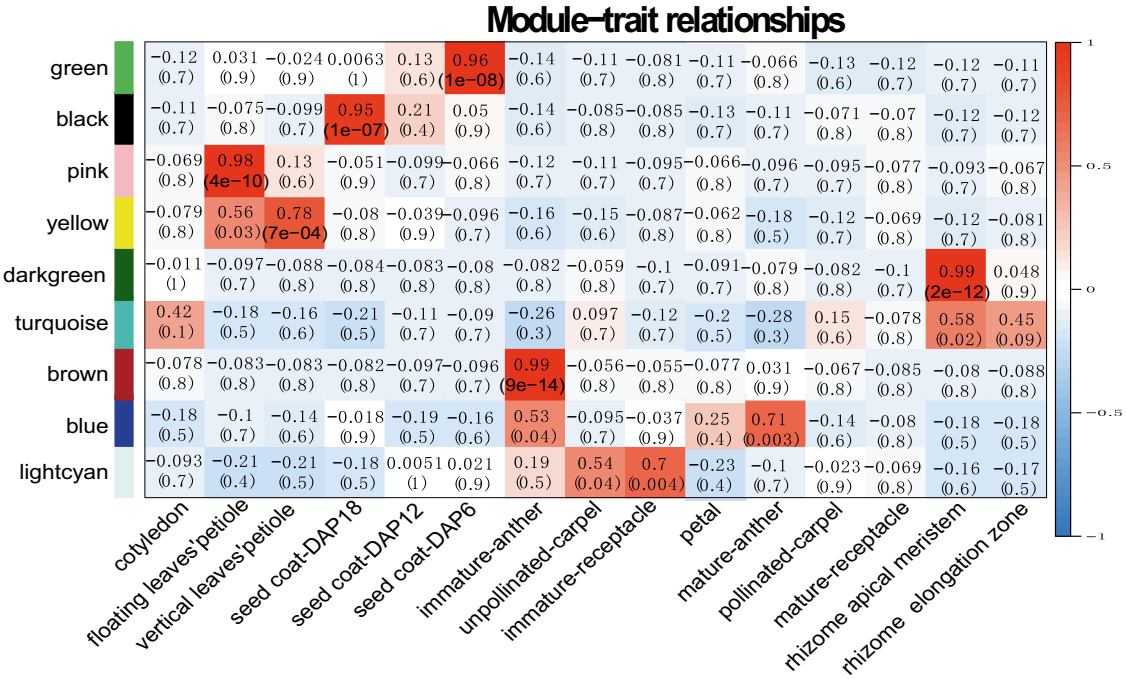
Module−trait relationships
green
black
pink
yellow
darkgreen
turquoise
brown
blue
lightcyan
cotyledon
floating leaves'petiole
vertical leaves'petiole
seed coat-DAP18
seed coat-DAP12
seed coat-DAP6
immature-anther
unpollinated-carpel
immature-receptacle
petal
mature-anther
pollinated-carpel
mature-receptacle
rhizome apical meristem
rhizome elongation zone
1
0.5
0
−0.5
−1

Discussion

Calcium, a universal second messenger, plays a key role in the signal transduction process during plant growth, development, and stress response (McAinsh and Pittman 2009). CaMs are conserved Ca^{2+} sensors in all eukaryotic cells. In addition to the CaMs, there are plant-specific calmodulins-like proteins (CMLs) that exist in plants (DeFalco et al. 2009). The previously reported genome sequence of the sacred lotus (Wang et al. 2015a; Ming et al. 2013) facilitates the study of genome-wide identification and expression analysis of *NNU-CaMs/CMLs*. In this study, we identified 34 *NNU-CaMs/CMLs* which showed extensive variations in gene length, theoretical molecular weight (Mw), and isoelectric point (pI) (Table 1), indicating the diversity of the *NNU-CaMs/CMLs*. The number of *NNU-CaMs/CMLs* was more than those in *Lotus japonicas* (7 LjCaMs and 19 LjCMLs), but less than those in *Arabidopsis* (6 AtCaMs and 50 AtCMLs), rice (3 OsCaMs and 32 OsCMLs), apple (4 MdCaMs and 58 MdCMLs), and grapevine (3 VviCaMs and 62 VviCMLs) (Liao et al. 2017; McCormack and Braam 2003; Boonburapong and Buaboocha 2007; Li et al. 2019; Vandelle et al. 2018). It may be that the incomplete genome sequencing and rigorous screening criteria lead to a small number of *NNU-CaMs/CMLs*. The predicted number of EF-hand in NNU-CaMs/CMLs ranged from one to four, which is consistent with those in rice (Boonburapong and Buaboocha 2007).

CaM proteins do not contain any palmitoylation or myristoylation signaling sequence (Mohanta et al. 2017, 2019), and the majority of their targets are cytosolic or nuclear proteins (Perochon et al. 2011). Therefore, CaM proteins are often found in the nucleus and cytoplasm (Wu et al. 2012; Zeb et al. 2020). The majority of CMLs also do not have localization signatures, and nuclear-cytoplasmic localization has been observed (Perochon et al. 2010; Trande et al. 2019). Meanwhile, CMLs were reported in various locations, such as AtCML30 localized in mitochondria (Chigri et al. 2012); AtCML36 (Astegno et al. 2017) and OsCML3 (Chinpongpanich et al. 2015) were localized in the plasma membrane. In this study, the NNU-CaMs/CMLs were predicted to mainly localize in the nucleus and cytoplasmic, and they were also predicted to be plastid proteins and plasma membrane proteins (Table 1), in consonance with the previous reports. The results of subcellular localization imply that NNU-CaMs/CMLs have diverse functions in the process of calcium signing transduction.

Based on the phylogenetic tree, CaMs/CMLs from *Arabidopsis*, rice, and lotus clustered within the same group and were classified into 12 subgroups (Fig. 1). NNU-CaMs/CMLs tightly clustered with AtCaMs/CMLs than with OsCaMs/CMLs, which was similar to those of woodland strawberry (*Fragaria vesca*) (Zhang et al. 2016), suggesting that CaMs/CMLs family is conserved in eudicots and monocots. Interestingly, *NNU-CaMs/CMLs* presented in Group 1, Group 2, and Group 8 contained several introns, while the other members only contained one exon (Fig. 2). In agreement with *AtCaMs/AtCMLs* and *OsCaMs/OsCMLs* (McCormack and Braam 2003; Boonburapong and Buaboocha 2007), most of *NNU-CaMs/CMLs* are intronless. In the evolutionary process of Ca^{2+} signing molecules, some calcium signaling genes were lost in the monocot lineage (Mohanta et al. 2019). In a previous study, FvCMLs were more clustered with AtCMLs than with OsCMLs (Zhang et al. 2016). The present study revealed Groups 7 and 8 only contained AtCMLs and NNU-CMLs, but no OsCMLs, probably because this cluster only represented CMLs in eudicots. These results indicated that the structure of *NNU-CaMs/CMLs* was diverse and conserved in eudicots, which may result in functional diversity.

The specific expression of genes is determined by the cis-acting elements in the promoter region. Previous studies have shown that certain *CML* genes with special cis-acting elements in the promoter region participated in hormonal or abiotic stress responses, such as *AtCML9* (Magnan et al. 2008) and *MtCML40* (Wang et al. 2019). In our study, *NNU-CaMs/CMLs* had various cis-acting elements related to plant growth and development, abiotic stress, plant hormones (Fig. 4). More than half of the *NNU-CaM/CML* genes contained cis-acting elements related to ABA (ABRE) and drought (MBS) stresses, indicating that *NNU-CaM/CML* genes may extensively participate in ABA and drought regulation. Half of the *NNU-CaM/CML* genes had anaerobic-responsive elements (ARE), which might be related to the adaptability to the aquatic environment.

The expressional patterns of *NNU-CaM/CML* genes in 15 different sacred lotus tissues had powerful specificity, which demonstrated that these genes are mainly involved in the regulation of initial floating leaves' petiole, immature-anther, and seed coat development processes (Fig. 5). The results clarify that *NNU-CaMs/CMLs* involved in the regulation of development. The variety of petiole rigidity produces two petioles types of lotus, initial floating leaves' petiole, and initial vertical leaves' petiole. A previous study showed the process of cell wall biosynthesis and lignin biosynthesis affects lotus petioles rigidity formation (Li et al. 2020). In this study, five *NNU-CaMs/CMLs* (NNU_07289, NNU_02678, NNU_12734, NNU_07897, NNU_16648) were highly expressed in initial floating leaves' petiole, implying that they may be involved in the regulation of cell wall biosynthesis, which was further supported by the observation that PdIQD10 interaction with two calmodulin proteins and involved in secondary cell wall biosynthesis in *Populus* (Badmi et al. 2018). Seven *NNU-CaMs/CMLs* showed high expression levels in the immature-anther, indicating that

they may be associated with early anther development in lotus. Furthermore, WGCNA had shown that *NNU-CaMs/CMLs* were divided into 11 modules and GO enrichment analysis of co-expressed genes in each module also demonstrated that these genes are involved in a wide range of biological processes such as regulation, post-translational protein modification, and the response to stress, of which each module was significantly correlated with development traits in lotus (Fig. 7). These results collectively indicated that *NNU-CaM/CML* genes might have undergone functional differentiation, participating in multiple growth and development processes and interacting with multiple genes to regulate physiological responses. It is especially special that the results of the module-trait relationships showed the leaves' petiole preferential expressed gene *NNU_12734* correlated with two petioles types in sacred lotus, which contain ARE cis-acting regulatory element essential for the anaerobic induction. Meanwhile, GO enrichment analysis indicated that NNU_12734 was involved in the cellular polysaccharide metabolic process. There is consistent data that NNU_12734 might participate in the pathway of petiole rigidity formation in lotus.

In light of calcium signal playing an important role in plant stress response, the expression patterns of *NNU-CaMs/CMLs* in response to calcium treatment may be useful in exploring its functional responsibility in calcium signaling. We analyzed the expression of 15 *NNU-CaMs/CMLs* in response to $CaCl_2$ and the chelating compounds EDTA by qRT-PCR. Approximate 60% *NNU-CaMs/CMLs* were up-regulated after 10 mM $CaCl_2$ treatment (Fig. 6), indicating that the high concentration of exogenous calcium ion stimulates cells and activates Ca^{2+}-permeable channels, increasing intracellular Ca^{2+} concentration to generate calcium signaling, and the expression of calcium-binding receptors NNU-CaMs/CMLs is elevated afterward. The *NNU-CaMs/CMLs* has a typical Ca^{2+} signaling process (Tian et al. 2020) after calcium treatment. The expression of *NNU-CaMs/CMLs* had no significant change after the early stage of 10 mM EDTA treatment, which chelate Ca^{2+}, Mg^{2+} by diamine, and four carboxylate groups (Clapham 2007), demonstrating that the low intracellular Ca^{2+} concentration would not induce *NNU-CaMs/CMLs* expression. These results indicated that the concentration of calcium ions will affect the NNU-CaMs/CMLs to perceive the calcium signaling in the lotus. Previous studies have suggested exogenous calcium reduced harmful effects caused by salt stress in *Phyllanthus amarus* (Jaleel et al. 2008), sunflower (*Helianthus annuus* L.) (Sohan et al. 1999), and rice (*Oryza sativa* L.) (Rahman et al. 2016; Roy et al. 2019). Therefore, *NNU-CaMs/CMLs* response to the change in exogenous calcium concentration can provide new avenues for further studies on *NNU-CaMs/CMLs* role in abiotic stress including excess ions.

The gene structure and conserved domain in specific proteins determine the molecular function involved in growth, development, and stress response, and genes in a designated family have a similar and differential function because of the variation in the promoter sequence and coding sequence. Thus, investigating the characterization of members in a gene family will improve our understanding of their potential function in regulating biological processes. In the present study, *NNU-CaMs/CMLs* gene family was analyzed based on genome sequencing, and the gene structure, protein domain, expression profiling, and the response to Ca^{2+} treatment were expounded. The results presented here could provide a theoretical basis on the molecular mechanism of *CaM/CML* genes family in lotus (*NNU-CaMs/CMLs*) in response to calcium signaling. Novel information obtained will contribute to further research on the effect of exogenous Ca^{2+} on stress tolerance, as well as to select potential candidate genes in *N. nucifera* for functional studies. In addition, this study will pave the way for developing cultivars with improved tolerance against high calcium environments in lotus.

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Author Contribution P. Y. designed the experiment. L.G. performed the experiments. L.G. and F. Y. analyzed the results and prepared the original manuscript. F. Y., D.N.R., and P. Y. revised the manuscript.

Declarations

Conflict of Interest The authors declare no competing interests.

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