Identification and expression analysis revealed drought stress-responsive *Calmodulin* and *Calmodulin-like* genes in maize

Zhen Wang*, Lihong Wang*, Jiaxin Li*, Wei Yang, Jiabin Ci, Xuejiao Ren, Wei Wang, Yingbai Wang, Liangyu Jiang, and Weiguang Yang

*College of Agriculture, Jilin Agricultural University, Changchun, People’s Republic of China; Crop Science Post-doctoral Station, Jilin Agricultural University, Changchun, People’s Republic of China

**ABSTRACT**

*Calmodulin* (CaM) and *Calmodulin-like* (CML) genes are the primary families of Calcium (Ca²⁺) sensors which are found to be involved in response to various stresses. Some genes involved in Ca²⁺ signal transduction have been genome-wide characterized in various species. However, the detailed identification, characterization, and expression profiles of *ZmCaM* and *ZmCML* genes in maize remain poorly understood, especially in the response to drought stress. In this study, a total of 7 *ZmCaMs* and 46 *ZmCMLs* are identified in maize and unevenly located on 10 chromosomes. *ZmCaM* and *ZmCML* proteins are divided into 9 groups. Protein structures analysis shows that the EF-hand motif number of *ZmCaMs/ZmCMLs* ranges from 3 to 4 and 2 to 4, respectively. A large number of cis-regulatory elements are found in the promoter regions of *ZmCaMs* and *ZmCMLs*. *ZmCaM* and *ZmCML* genes display highly diversified tissue-specific expression patterns. Furthermore, *ZmCaM2, ZmCML3, ZmCML6, ZmCML8, ZmCML19, ZmCML24, ZmCML27, ZmCML28, ZmCML36, ZmCML39*, and *ZmCML40* are induced significantly under drought stress through RT-PCR analysis. Taken together, these results will help to understand the critical roles of *ZmCaM* and *ZmCML* genes played in drought resistance and provide valuable candidate genes that could be used to develop drought-resistant maize.

**Introduction**

Plants are subjected to various abiotic and biotic stresses in the process of growth and development, such as pathogens, low or high temperature, and drought stresses (Zhu 2016). In order to adapt to stress conditions, plants have evolved multiple biochemical and physiological processes, including plant hormone regulation and cell signal transduction (Shukla et al. 2019; Berens et al. 2017). Calcium (Ca²⁺) as an important second messenger plays a key role in the transmission and amplification of cell signals (La Verde et al. 2018). A wide range of external stimuli can induce rapid and transient changes in cytosolic Ca²⁺ concentration which are recognized by several types of Ca²⁺ binding proteins (Hashimoto and Kudla 2011). Typically, Ca²⁺ binding proteins can be divided into four major groups, including calmodulin (CaM), calmodulin-like protein (CML), Ca²⁺ dependent protein kinase (CDPK), and calcineurin-B-like protein (CBL) (Heyer et al. 2008; Aldon et al. 2018; Wang et al. 2019). The majority of Ca²⁺ binding proteins have EF-hand motifs which are found to bind Ca²⁺. CaM is a highly conserved Ca²⁺ binding protein that is composed of approximately 150 amino acid residues with four EF-hand motifs (Chin and Means 2000). Although CaM has no enzymatic function of its own, it can transduce the signals by interacting with other target proteins (Perochon et al. 2011). CMLs are a large subgroup of Ca²⁺ binding proteins which normally possess 1–6 EF-hand motifs (Hashimoto and Kudla 2011). Previous studies have shown that CaMs and CMLs do not have any functional domains except for EF-hand motifs (Zhu et al. 2015). At present, CaMs and CMLs have been identified in many plant species. For example, 7 CaMs and 50 CMLs have been identified in the whole genome of *Arabidopsis* (McCormack and Braam 2003), 5 CaMs and 32 CMLs have been identified in rice (Boonburapong and Buaboocho 2007), 6 CaMs and 144 CMLs have been identified in soybean (Zeng et al. 2017), and 6 CaMs and 45 CMLs have been identified in *Solanum lycopersicum* (Munir et al. 2016).

CaM and CML genes have been shown to be involved in plant developmental process and response to various stresses. Overexpression of *AtCaM1* and *AtCaM4* in *Arabidopsis* results in enhanced leaf senescence (Dai et al. 2018; Koo et al. 2017). *AtCML24* and *AtCML25* are involved in pollen germination and pollen tube growth (Yang et al. 2014; Wang et al. 2015). *AtCaM2* and *AtCaM7* play vital roles in pollen germination and seedling development, respectively (Landoni et al. 2010; Kushwaha et al. 2008). The knockout mutants of *AtCML9* display enhanced tolerance to drought and salinity stresses (Magnan et al. 2008). *AtCML20* is a negative regulator of ABA-induced stomatal movement and drought stress tolerance (Wu et al. 2017). *AtCML39* positively regulates...
ABA-mediated signaling pathway and negatively regulates GA3-mediated signaling pathway (Midhat et al. 2018). OsCML4, OsCML5, OsCML8, and OsCML11 in rice are involved in osmotic and salt stresses (Chinpongpanich et al. 2012). OsCML16 positively regulates root growth and drought tolerance under the regulation of transcription factor OsERF48 (Jung et al. 2017). Transgenic tomato plant overexpressing ShCML44 shows increased tolerance to drought and low-temperature stresses (Munir et al. 2016). Overexpression of GsCaM27 in Arabidopsis confers enhanced bicarbonate tolerance, while it decreases salt and osmotic tolerance during seed germination and early growth (Chen et al. 2015). The expression level of TaCaM20 is regulated by drought stress, and TaCaM20 overexpression transgenic wheat lines enhance water-soluble carbohydrate accumulation (Kalaipandian et al. 2019). VaCML21 positively regulates the plant tolerance to cold stress through increasing the expression of the cold stress-responsive marker genes (Aleyanova et al. 2020). In addition, CaM and CML genes are found to be involved in response to pathogen infection. The expression level of AtCML8 is induced after infection with Pseudomonas syringae (Zhu et al. 2017). In cotton, GhCaM/L and GhCML11 positively participate in defense reaction to Verticillium dahliae (Cheng et al. 2016).

Maize (Zea mays) is an important food and economic crop on earth, but its production is seriously constrained by drought stress. Ca2+ signals play significantly important roles in stress tolerance in maize (Li et al. 2004), and some genes (CAMTA) involved in Ca2+ signal transduction have been genome-wide characterized (Yue et al. 2015). However, detailed characterization and expression patterns of ZmCaM and ZmCML genes family in maize are largely unknown, especially their potential roles in response to drought stress. Here, we identified 7 ZmCaM and 46 ZmCML genes in the maize B73 genome and performed comprehensive bioinformatics analysis, including molecular characterization, gene structure, phylogenetic classification, chromosome location, and cis-regulatory elements. In addition, the expression levels of ZmCaM and ZmCML genes in different tissues and in response to drought stress were analyzed based on RNA-seq database and real-time quantitative PCR (RT-qPCR). These results will help us to understand the functional characterization of ZmCaM and ZmCML genes in response to drought stress in maize.

Materials and methods

Plant materials and stress treatments

The seeds of maize inbred line B73 were provided by Maize Breeding Innovation Team of Jilin Agricultural University and were collected in accordance with relevant national regulations. The seeds of maize were sterilized with 6% sodium hypochlorite for 10 min and rinsed with distilled water. The seeds were grown in germination box for 4–5 days with 16 h light (28°C) and 8 h dark (21°C). For drought treatment, the seedlings were soaked with Polyethylene glycol (PEG) 8000 at a water potential of −0.2 MPa (moderate stress) and −0.8 MPa (severe stress) when the length of the main root reached 2–4 cm (Opitz et al. 2014). The mock-treated plants were treated with sterile water. The roots were sampled at 0, 6, and 24 h, respectively. The whole roots were frozen in liquid nitrogen immediately and stored at −80°C until RNA was extracted. The experiment was performed using three biological replicates with three technical replicates.

Identification of the ZmCaM and ZmCML genes in maize

For the search of ZmCaM and ZmCML proteins by BLASTP in B73-Reference-Grap-hene-4.0 genome database (http://alpha.maizegdb.org/), 9 AtCaM and 45 AtCML proteins were used as query sequences. The non-ZmCaM and ZmCML sequences were filtered by NCBI CDD (https://academic.oup.com/nar/article/45/D1/D200/2605748), SMART (https://academic.oup.com/nar/article/46/D1/D493/4429069), and interpro (http://www.ebi.ac.uk/interpro). The amino acids (AA), theoretical isoelectric point (pl), molecular weight (MW), protein grand average of hydropathicity (GRAVY), and protein location of ZmCaM and ZmCML protein sequences were analyzed by Expasy (https://web.expasy.org/compute_pl/), Protein Gravy (http://www.databio.com/sms2/protein_gravy.html), and Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/), respectively.

Gene structure and phylogenetic analysis

The exon-intron structure of ZmCaM and ZmCML genes were analyzed by GSDS2.0 (https://gsds.cbi.pku.edu.cn/). The EF-hand motif was characterized using MEME (http://meme-suite.org/tools/meme) and SMART (http://smart.embl-heidelberg.de/). Sequence alignment of CaM/CML proteins in Arabidopsis, rice, and maize were performed by mafft (https://academic.oup.com/mbe/article-lookup/doi/10.1093/molbev/msst010). The phylogenetic tree was constructed and modified by protest (https://www.ncbi.nlm.nih.gov/pubmed/21335321), RAXML (https://www.ncbi.nlm.nih.gov/pubmed/24451623), and iTOL (https://itol.embl.de/).

Chromosomal location and synteny analysis of ZmCaM and ZmCML genes

ZmCaM and ZmCML genes were mapped to maize chromosomes by mg2c (http://mg2c.iask.in/mg2c_v2.0/), and the map was drafted using Mapchart software (https://www.wageningen.ur.nl/en.htm). The nonsynonymous substitution rate (Ka) and synonymous substitution rate (Ks) among homologous gene pairs were calculated. There were three directions of gene evolution: positive selection (Ka >> Ks or Ka/Ks >> 1), neutral evolution (KA = Ks or Ka/Ks = 1) and purify selection (KA << Ks or Ka/Ks << 1) (Li et al. 2009).

Analysis of cis-regulatory elements of ZmCaM and ZmCML genes

The promoter regions up to 2000 bp in the ZmCaMs and ZmCMLs genes were used to analyze potential cis-regulatory elements by the online tool PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Expression pattern analysis of ZmCaM and ZmCML genes

To identify the expression patterns of ZmCaM and ZmCML genes in different tissues, the transcription data of ZmCaM and ZmCML genes were derived from Walley’s report...
(Walley et al. 2016). The RNA-seq data for maize roots with low water potentials were downloaded from the NCBI Gene Expression Omnibus under accession PRJNA226757 (Opitz et al. 2014). FPKM values were used to compare ZmCaMs and ZmCML expression levels.

**RT-qPCR assay**

RNA was extracted by the method of TRIZol and was reverse transcribed into cDNA by TOYOBO kit. RT-qPCR was analyzed using the SYBR Green Supermix kit (Genstar) according to the instruction manual with QuantStudio 3. The housekeeping gene of maize ZmTub (GRMZM2G066191) was used as an internal control to normalize the data. The relative expression level was calculated using the 2ΔΔCT method. The experiment was performed using three biological replicates with three technical replicates, and was statistically analyzed using Student’s t-test (*p < 0.05, **p < 0.01). Bars indicate the standard error of the mean. The primers used in the experiment are shown in Table S4.

**Gene interaction network of the ZmCaM/ZmCML proteins**

The interaction network of ZmCaM/ZmCML proteins was analyzed using online STRING database (https://string-db.org/).

**Results**

**A total of 53 ZmCaMs and ZmCMLs are identified in maize**

To identify the ZmCaM and ZmCML genes in the maize genome, the AtCaM and AtCML genes of Arabidopsis were used as query sequences. A total of 53 ZmCaMs/ZmCMLs were identified, including 7 ZmCaMs and 46 ZmCMLs, which were named ZmCaM1-ZmCaM7 and ZmCML1-ZmCML46, respectively. The coding sequences of ZmCaMs/ZmCMLs were analyzed, including AA, pI, MW, GRAVY, and protein location. The sizes of ZmCaMs/ZmCMLs ranged from 80 (ZmCML2 and ZmCML23) to 398 (ZmCaM5) AA residues. The average molecular weight of ZmCaMs/ZmCMLs was 20.60 kDa, varying between 8.77 (ZmCML23) and 44.88 (ZmCaM5) kDa. The pI of these proteins ranged from 4.17 to 4.87 and 3.89 to 9.12, respectively. Hydrophilicity analysis showed that most ZmCaMs/ZmCMLs are hydrophilic proteins, except ZmCML10, ZmCML29, ZmCML30 and ZmCML32. To further investigate the potential functions of ZmCaMs/ZmCMLs, the subcellular localization was predicted using Cell-PLoc 2.0. We found that all 7 ZmCaM proteins were predicted to be in the cell membrane and cytoplasm. The ZmCML proteins were predicted to be in different organelles, including the nucleus, vacuole, and cell membrane (Table S1). These results indicate that identified ZmCaMs/ZmCMLs may have diverse functions in Ca2+ signaling transduction.

**ZmCaMs and ZmCMLs are divided into nine groups and carry different numbers of the EF-hand motif**

To gain insights into the potential functions of ZmCaMs/ZmCMLs, the phylogenetic tree was built using the CaM/CML protein sequences from Arabidopsis, rice, and maize, respectively. The phylogenetic tree showed that ZmCaMs/ZmCMLs are divided into nine groups, namely groups I–IX, according to their similarities and relationships with Arabidopsis and rice members (Shi and Du 2020; Sun et al. 2020). Groups I–IX contained 2, 3, 12, 4, 3, 9, 7, 3, and 11 members, respectively. All seven ZmCaMs were divided into group I (Figure 1). These results demonstrate that CaM/CML proteins from Arabidopsis, rice, and maize show highly homologous.

EF-hand motif is the main domain of calcium-binding protein, which is found to be involved in binding Ca2+. As shown in Figure 2(A), the EF-hand motif number of ZmCaMs/ZmCMLs ranged from 3 to 4 and 2 to 4, respectively. In total, 17, 16, and 12 ZmCMLs were found to have 4, 3, and 2 EF-hand motifs, respectively. In addition, ZmCaM2 had four EF-hand motifs, the remaining ZmCaMs contained 3 EF-hand motifs. These results demonstrate that all identified ZmCaM and ZmCML proteins carry typical EF-hand domains, which may have function in binding Ca2+. The different numbers of the EF-hand motif may lead to functional differences among ZmCaMs/ZmCMLs.

The gene structures of 53 ZmCaMs/ZmCMLs were analyzed based on the arrangement of exon/introns. The majority of the ZmCaMs/ZmCMLs lacked introns, while 1–7 introns were found in 21 ZmCaMs/ZmCMLs. ZmCaM3, ZmCML17, ZmCML27, ZmCML46, ZmCaM3, ZmCaM6, and ZmCaM7 had 1 intron, while ZmCML19 and ZmCML39 contained the largest number of introns, up to 7 (Figure 2B). These results show that the number of introns in ZmCaMs/ZmCMLs is different, which may exist in gene structure differentiation in the evolution process.

**ZmCaMs and ZmCML genes are unevenly distributed on 10 chromosomes**

To determine the distribution of ZmCaMs/ZmCMLs in maize genome, their chromosomal location was analyzed by mg2c (http://mg2c.iask.in/mg2c_v2.0/). The results demonstrated that 7 ZmCaMs and 46 ZmCMLs are distributed on 10 chromosomes, but the distribution appears to be unbalanced. Most ZmCaM and ZmCML genes were located on chromosome 2, 3, and 4, whereas a single ZmCML4 was found on chromosome 10. There are only four genes each on chromosome 1, 5, 7, 8, and 9. In addition, six genes (ZmCaM3, ZmCaM4, ZmCML34, ZmCML35, and ZmCML37) were located on chromosome 6 (Figure 3). The uneven distribution of ZmCaMs/ZmCMLs demonstrates that genetic variations exist in the process of evolution.

**ZmCaMs and ZmCML genes experience purification selection**

Previous studies have shown that gene duplication can lead to generating new functional genes and drive species evolution (Panchy et al. 2016). We performed synteny analysis for exploring duplications within the ZmCaMs and ZmCMLs family. As shown in Figure 4, most of ZmCaMs/ZmCMLs were found to exist segmental-duplication gene pairs, except ZmCML6 and ZmCML19. The nonsynonymous substitution rate (Ks) and the synonymous substitution rate (Ka) represented the selection...
Figure 1. Phylogenetic analysis among CaM and CML proteins of maize, rice, and Arabidopsis. The phylogenetic trees of CaM and CML proteins in maize, rice, and Arabidopsis were divided into nine groups. Red represents the CaM and CML proteins in Arabidopsis. Blue represents the CaM and CML proteins in rice. Green represents the CaM and CML proteins in maize.

Figure 2. ZmCaM and ZmCML genes have different structures and carry different numbers of the EF-hand motif. (A) The number of EF-hand motif in ZmCaM and ZmCML proteins. (B) The structure of ZmCaM and ZmCML genes. Blue, gray lines, and yellow indicate CDS, introns, and untranslated regions, respectively.
and evolution directions of protein-coding genes (Li et al. 2009). There were three directions of gene evolution: positive selection (\( \text{Ka/Ks} >> 1 \)), neutral evolution (\( \text{Ka/Ks} = 1 \)), and purify selection (\( \text{Ka/Ks} << 1 \)). Table S2 showed that the average Ka/Ks ratio of 172 collinear gene pairs is 0.20. These results indicate that \( \text{ZmCaM} \) and \( \text{ZmCML} \) genes experience purification selection.

More than 110 cis-regulatory elements are found in the promoter regions of \( \text{ZmCaM} \) and \( \text{ZmCML} \) genes

To investigate the function of \( \text{ZmCaMs/ZmCMLs} \), 2000 bp sequences of promoter regions for the \( \text{ZmCaMs/ZmCMLs} \) were analyzed using the PlantCARE tool. As shown in Table S3, more than 110 cis-regulatory elements were found in the promoter regions of \( \text{ZmCaMs/ZmCMLs} \). They were classified into five categories according to different functions, including core promoter element, light response element, hormone response element, stress response element, and other regulatory element. The cis-regulatory elements were involved in hormone response including auxin (AuxRE and AuxRR-core), gibberellin (GARE-motif and TATC-box), ethylene (ERE), salicylic acid (SARE and TCA-element), and MeJA (TGACG-motif). Stress response elements are mainly composed of hypoxia-induced response element (ARE), drought response element (MBS), dehydration response element (DRE), low-temperature response element (LTR), trauma-induced response element (WUN-motif), pressure response element (STRE), defense and stress response elements (TC-rich repeats). These results indicate that \( \text{ZmCaMs/ZmCMLs} \) may respond to various stresses.

ZmCaM and ZmCML genes have different expression patterns in various tissues

To further reveal potential function of \( \text{ZmCaMs/ZmCMLs} \) in maize, the transcription levels of \( \text{ZmCaMs/ZmCMLs} \) were analyzed in different tissues via RNA-seq database (Walley et al. 2016). As shown in Figure 5, most of \( \text{ZmCaMs/ZmCMLs} \) were expressed in endosperm, leaf, root, and other tissues, whereas some \( \text{ZmCMLs} \) (ZmCML9, ZmCML14, ZmCML33, ZmCML43 and ZmCML44) were not expressed in tissues. ZmCML2, ZmCML4, ZmCML8, ZmCML11, ZmCML20, ZmCML23, ZmCML25, ZmCML27, and ZmCML40 showed high expression levels in maize natural pollination. ZmCML10, ZmCML21, ZmCML26, ZmCML30, ZmCaM1, ZmCaM7, ZmCML44, and ZmCML35 were highly expressed in root elongation zone and cortex. ZmCML6, ZmCML32, ZmCML19, and ZmCML39 had high expression profiles in mature leaves and leaf zone. Interestingly, we also found that some \( \text{ZmCaMs/ZmCMLs} \) displayed different expression patterns in the developmental process of leaf and roots (Figure 5). The expression profiles of \( \text{ZmCaMs/ZmCMLs} \) in different tissues demonstrate that \( \text{ZmCaMs/ZmCMLs} \) may have different biology functions in plant developmental processes.

ZmCaM and ZmCML genes are regulated under drought stress

To illuminate the possible functions of \( \text{ZmCaMs/ZmCMLs} \) in response to drought stress, their expression patterns were analyzed in maize roots under drought stress via RNA-seq database (Li et al. 2009). RNA-seq database revealed that most of \( \text{ZmCaMs/ZmCMLs} \) are regulated under drought stress, except ZmCML4 and ZmCML9. As shown in Figure 6, the expression levels of ZmCML12, ZmCML22, and ZmCML30 were up-regulated under moderate stress and severe stress at 6 h. The expression patterns of ZmCML3, ZmCML8, and ZmCML26 were up-regulated under moderate stress and severe stress at 24 h. The expression patterns of ZmCaM7 and ZmCML10 were down-regulated at 6 h under moderate stress and severe stress. The expression levels of ZmCaM5 and

Figure 3. \( \text{ZmCaM} \) and \( \text{ZmCML} \) genes are unevenly distributed on 10 chromosomes.
ZmCML2 were higher under moderate stress at 24 h than under severe stress at 24 h. The expression levels of ZmCML14 and ZmCML38 were increased under moderate stress at 24 h, while decreased under severe stress at 24 h. These results suggest that ZmCaMs/ZmCMLs have distinct expression patterns under moderate and severe stresses, which may play different roles in drought tolerance.

To confirm the results of RNA-seq, the expression levels of ZmCaMs/ZmCMLs (ZmCaM2, ZmCML3, ZmCML6, ZmCML8, ZmCML19, ZmCML24, ZmCML27, ZmCML28, ZmCML36, ZmCML39, and ZmCML40) were detected in maize under drought stress using RT-qPCR. As shown in Figure 7, the expression levels of ZmCaMs/ZmCMLs were strongly induced in maize roots under drought stress, which were consistent with RNA-seq data. The relative expression level of ZmCML39 was significantly increased under moderate drought stress at 6 h, while was decreased under severe drought stress at 6 h. The relative expression levels of ZmCML3 and ZmCML8 were increased under moderate and severe drought stress at 24 h. In addition, we also found that ZmCaM2, ZmCML3, ZmCML6, ZmCML8, ZmCML19, ZmCML24, ZmCML28, ZmCML36, ZmCML39, and ZmCML40 have the highest expression levels under severe drought stress at 24 h, which indicate that they are responsive to severe drought stress. These results indicate that identified ZmCaMs/ZmCMLs may play important roles in drought resistance.

The interaction network of ZmCaM and ZmCML proteins

Interaction network analysis is helpful to study the function of genes and discover the core regulatory genes (Zhao et al. 2018). The relative expression levels of ZmCaM2, ZmCML3, ZmCML8, and ZmCML40 reached the highest under severe drought stress at 24 h, which indicate they may play important roles in response to drought stress. To further explore the function of these genes, we conducted the network interaction analysis using STRING database. As shown in Figure 8, ZmCaM2, ZmCML3, ZmCML8, and ZmCML40 proteins were associated with 10 functional proteins, respectively. The cal2 (homologous protein of ZmCaM2) was associated with plant defense signaling network protein (CBL6 and Si496006g11), which plays roles in transmitting ROS signals (Figure 8A) (Sewelam et al. 2016). The GRMZM2G149923_P01 (homologous to ZmCML3) was associated with CBL proteins, which participate in response to drought stress (Figure 8B) (Moumeni et al. 2011). Similarly, the GRMZM2G115628_P01 (homologous to ZmCML8) was predicted to be interacting with CBL1, which is a positive regulator of salt and drought stresses (Figure 8C) (Cheong et al. 2003). In addition, the pco068771 (homologous to ZmCML40) was predicted to be interacting with GRMZM2G120922_P01, which plays crucial roles in maize root under drought stress (Figure 8D) (Zaidi et al. 2016). These results indicate that ZmCaM2, ZmCML3, ZmCML8, and ZmCML40 proteins...
may regulate the maize drought resistance through interaction with these proteins.

**Discussion**

*Identification and characterization of ZmCaM and ZmCML genes*

Plants have evolved a series of physiological and biochemical mechanisms to cope with stress during evolution. Previous studies have shown that Ca²⁺ is a crucial second messenger in eliciting response to environmental stimuli. CaMs and CMLs as conserved Ca²⁺ sensor proteins involved in Ca²⁺ signaling transduction in plants (Cheval et al. 2013). However, the comprehensive identification and expression of ZmCaM/ZmCML genes in maize are largely unclear, especially in the response to drought stress. (Mohanta et al. 2017) have studied the evolution of CaM and CML genes in various species, including in maize (Mohanta et al. 2017). In Mohanta’s report, only 8 ZmCaMs and 21 ZmCMLs are identified in maize, however, the characterization and potential roles of these genes are unclear. In this study, more detailed ZmCaM/CML genes (7 ZmCaMs and 46 ZmCMLs) were identified in maize through BLASTP in B73-Reference-Graphene-4.0 genome database (http://alpha.maize-gdb.org/) (Table S1). Furthermore, a comprehensive analysis of ZmCaM/CML genes was accomplished including subcellular localization phylogenetic tree, conserved motifs, exon-intron structure, chromosome location, syntenic analysis, cis-regulatory elements, expression patterns in various tissues and under drought stress and interaction network. These results will provide novel and valuable information that will help understand the function and regulatory mechanism of ZmCaM/CML genes in maize, especially in drought resistance.

Previous studies showed that CaM and CML proteins contain the conserved EF-hand motif which is a key Ca²⁺ binding site and the analysis of gene structure and conserved motifs can help us to understand the function of gene family (Nelson et al. (2002)). Here, three to four typical EF-hand motifs were identified in ZmCaMs, and two to four typical EF-hand motifs were identified in ZmCMLs (Figure 2A). Gene structure analysis showed that most ZmCMLs lack introns, while ZmCaMs contain 1, 2, or 6 introns (Figure 5).
These findings demonstrate that the conservation and divergence of motif and intron numbers may lead to similar or different biological functions among ZmCaM/CML family members. In principle, evolving more genes are the correct direction for the plant to adapt to different environments (Li et al. 2019). More ZmCML genes in the maize genome indicate that ZmCML genes may evolve before ZmCaM genes (Figure 1).

The cis-elements in the promoters of ZmCaM and ZmCML genes

CaM and CML genes play critical roles in the plant developmental process and response to various stresses. These functions are associated with the cis-regulatory elements which are present in the promoter regions. Various hormone response elements and stress response elements have been
identified in the promoter regions of CaM and CML genes in plants (He et al. 2020). The LTRs were enriched in the promoter regions of MtCML16 and MtCML33, and the transcript expression levels of MtCML16 and MtCML33 were induced under cold treatment at 6 h (Sun et al. 2020). The transcriptional expression levels of OsCML4, OsCML5, and OsCML11 were significantly increased under dehydration (Chinpongpanich et al. (2012)). In this study, the AuxRE, GARE-motif, ERE, TCA-element, MBS, DRE, LTR, and TC-rich repeats were found in the promoter regions of ZmCaMs/ZmCMLs (Table S3). We speculate that they may involve in response to abiotic stresses such as low temperature, drought, and salt stresses.

**Expression profiles of ZmCaM and ZmCML genes in various maize tissues**

CaM and CML genes were reported to be diversely expressed in various plant tissues. MdCaM2 was expressed in all apple tissues, while MdCML7 and MdCML49 showed high expression in roots (Li et al. 2019). BrCML2.1, BrCML15.1, BrCML15.2, BrCML28.1, BrCML25.3, and BrCML28.3 were only expressed in flower (Guo et al. 2018). In tomato, SICML15, SICML46, SICML24, SICML20, SICML19, and SICML14 were expressed in various tissues, including young leaves and flowers (Munir et al. 2016). In addition, VviCML9a, VviCML9b, and VviCML79 were specifically expressed in senescent leaves, roots and fruit setting rate, respectively (Vandelle et al. 2018). In this study, we found that most ZmCaMs/ZmCMLs were expressed in plant tissues, except ZmCML9, ZmCML14, ZmCML33, ZmCML43, and ZmCML44 (Figure 5). For instance, ZmCML3, ZmCML29, and ZmCML45 were highly expressed in endosperm, indicating that these genes may be involved in endosperm synthesis. ZmCML16, ZmCML12, ZmCML41, ZmCML37, ZmCML17, ZmCML13, ZmCML42, ZmCML36, ZmCML30, ZmCML3, ZmCML1, ZmCML46, and ZmCML24 showed high expression in embryos, indicating that these genes may play a crucial role in the development of embryos. These results demonstrate that ZmCaMs/ ZmCMLs may play important and different roles in the process of plant growth and development.

---

**Figure 7.** Expression profiles of 11 ZmCaM and ZmCML genes were regulated under drought stress by RT-qPCR. RT-qPCR data were normalized using maize Actin gene. X-axes represent various treatments and different times (CK, control; −0.2MPA and −0.8MPA indicate degrees of drought treatment in roots). Y-axes are scales of relative expression level. A: ZmCaM2. B: ZmCML3. C: ZmCML6. D: ZmCML8. E: ZmCML19. F: ZmCML24. G: ZmCML27. H: ZmCML28. I: ZmCML36. J: ZmCML39. K: ZmCML40. The relative expression is calculated using the 2−ΔΔCt method. STDEV is used to calculate the standard deviation of sample-based estimates (*p < 0.05, **p < 0.01, Student’s t-test). The experiment was performed using three biological repeats.
Expression profiles of ZmCaM and ZmCML genes under drought stress

Previous studies demonstrated that CaM and CML genes as key regulators are involved in drought stress. AtCML20 played a negative role in drought tolerance, and overexpression of AtCML20 led to hypersensitive to drought stress (Wu et al. 2017). AtCML37 and AtCML42 responded to drought stress, and they interacted with each other under drought stress (Scholz et al. 2015). MsCML46 was localized in the cytoplasm and its expression was significantly increased under drought treatment (Du et al. 2021). Under water deficit condition, the expression levels of VaCML57, VaCML66, and VaCML88 were significantly decreased (Dubrovina et al. 2019). Rice Calmodulin-like gene OsMSR2 enhanced drought resistance in Arabidopsis thaliana (Xu et al. 2011). Rice Calmodulin-like gene OsMSR2 enhanced drought resistance in Arabidopsis thaliana (Xu et al. 2011). To further understand whether ZmCaMs and ZmCMLs respond to drought stress, their expression patterns were analyzed through RNA-seq and RT-qPCR. The relative expression levels of ZmCaM2, ZmCML3, ZmCML6, ZmCML8, ZmCML19, ZmCML24, ZmCML27, ZmCML28, ZmCML36, ZmCML39, and ZmCML40 were induced significantly under drought stress (Figures 6 and 7). Furthermore, drought stress-responsive elements are found in the promoter regions of 11 ZmCaMs and ZmCMLs genes. These results indicate that they may have critical functions in drought stress.

Conclusion

CaMs and CMLs protein are primary Ca$^{2+}$ binding protein family, which are involved in response to various stresses. In this study, we identified 7 ZmCaM and 46 ZmCML genes in maize, and they were unbalanced distributed on 10 chromosomes. Phylogenetic analysis showed that ZmCaMs/ZmCMLs are divided into nine subgroups. Gene structure analysis revealed that the EF-hand motif number of ZmCaMs/ZmCMLs ranged from 3 to 4 and 2 to 4, respectively. ZmCaMs/ZmCMLs showed different expression patterns among various tissues. A total of 11 ZmCaMs/ZmCMLs were significantly induced under drought stress based on RNA-seq data and qRT-PCR, which indicate that they may play crucial roles in drought resistance. Overall, these results may provide an in-depth understanding of the roles of ZmCaM/ZmCML genes played in drought resistance.

Author contributions

L. Y. J. and W. G. Y conceived and designed the experiments. Z. W., L. H. W., and J. X. L. performed the experiments and drafted the manuscript. W. Y., J. B. C., X. J. R., W. W., and Y. B. W. analyzed the data.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Youth Growth Technology Project of Science and Technology Department of Jilin Province, China (20210508020RQ), Innovation and Entrepreneurship Training Program for College Students in Jilin Province (S202110193120, S202110193125).
References

Aldon D, Mbengue M, Mazars C, Galaud JP. 2018. Calcium signaling in plant biotic interactions. Int J Mol Sci. 19(3):665.

Aleyanova OA, Kiselev KV, Ogneva ZV, Dubrovina AS. 2020. The grapevine calmodulin-like protein gene CML21 is regulated by alternative splicing and involved in abiotic stress response. Int J Mol Sci. 21(7):2593.

Berens ML, Berry HM, Mine A, Argueso CT, Tsuda K. 2017. Evolution of hormone signaling networks in plant defense. Annu Rev Phytopathol. 55:401–425.

Boonburarpong B, Buaboocha T. 2007. Genome-wide identification and analyses of the rice calmodulin and related potential calcium sensor proteins. BMC Plant Biol. 7:4.

Chen C, Sun X, Duannu H, Zhu D, Yu Y, Cao L, Liu A, Jia B, Xiao J, Zhu Y. 2015. GsCML27, a gene encoding a calcium-binding EF-hand protein from glycine soja, plays differential roles in plant responses to bicarbonate, salt and osmotic stresses. PLoS One. 10(11):e014888.

Cheng HQ, Han LB, Yang CL, Wu XM, Zhong NQ, Wu JH, Wang FX, Wang HY, Xia GX. 2016. The cotton MYB108 forms a positive feedback regulation loop with CML11 and participates in the defense response against Verticillium dahliae infection. J Exp Bot. 67(6):1933–1950.

Cheong YH, Kim KN, Pandey GK, Gupta R, Grant JJ, Luan S. 2008. CalB1L, a calcium sensor that differentially regulates salt, drought, and cold responses in Arabidopsis. Plant Cell. 15(8):1833–1845.

Cheval C, Aldon D, Galaud JP, Ranty B. 2013. Calcium/calmodulin-mediated regulation of plant immunity. Biochim Biophys Acta. 1837(3):1766–1771.

Chinpongpanich A, Limruengroj K, Phean-O-Pas S, Limpaseni T, Kushwaha R, Singh A, Chattopadhyay S. 2008. Calmodulin 7 plays an important role as transcriptional regulator in Arabidopsis seedling development. Plant Cell. 20(7):1747–1759.

Landoni M, De Francesco A, Galbiati M, Tonelli C. 2010. A loss-of-function mutation in Calmodulin2 gene affects pollen germination in Arabidopsis thaliana. Plant Mol Biol. 74(3):235–247.

La Verde V, Dominici P, Astegno A. 2018. Towards understanding plant calcium signaling through calmodulin-like proteins: A biochemical and structural perspective. Int J Mol Sci. 19(5):1331.

Li B, Liu HT, Sun DY, Zhou RG. 2004. Ca(2+)- and calmodulin modulate DNA-inking activity of maize heat shock transcription factor in vitro. Plant Cell Physiol. 45(6):627–634.

Li C, Meng D, Zhang J, Cheng L. 2019. Genome-wide identification and expression analysis of calmodulin and calmodulin-like genes in apple (Malus × domestica). Plant Physiol Biochem. 139:600–612.

Li J, Zhang Z, Vang S, Yu J, Wong GK, Wang J. 2009. Correlation between Ca/ks and Ks is related to substitution model and evolutionary lineage. J Mol Evol. 68(4):414–423.

Magnan F, Ranty B, Charpenteau M, Sotta B, Galaud JP, Aldon D. 2008. Mutations in AtCML9, a calmodulin-like protein from Arabidopsis thaliana, alter plant responses to abiotic stress and abscisic acid. Plant J. 56(4):575–589.

Mc Cormack E, Braam J. 2003. Calmodulins and related potential calcium sensors of Arabidopsis. New Phytol. 159(3):585–598.

Midhat U, Ting MKY, Teresinski HJ, Snedden WA. 2018. The calmodulin-like protein, CML39, is involved in regulating seed development, germination, and fruit development in Arabidopsis. Plant Mol Biol. 90(4–5):375–392.

Mohanta TK, Kumar P, Bae H. 2017. Genomics and evolutionary aspect of calcium signaling event in calmodulin and calmodulin-like proteins in plants. BMC Plant Mol. Biol. Feb 3(17):1–38.

Moumeni A, Satoh K, Kondoh H, Asano T, Hosaka A, Venuprasad R, Serra R, Kumar A, Leung H, Kikuchi S. 2011. Comparative analysis of root transcriptome profiles of two pairs of drought-tolerant and susceptible rice near-isogenic lines under different drought stress. BMC Plant Biol. 11:174.

Munir S, Khan MR, Song J, Munir S, Zhang Y, Ye Z, Wang T. 2016. Genome-wide identification, characterization and expression analysis of calmodulin-like (CML) proteins in tomato (Solanum lycopersicum). Plant Physiol Biochem. 102:167–179.

Munir S, Liu H, Xing Y, Hussain S, Ouyang B, Zhang Y, Li H, Ye Z. 2016. Overexpression of calmodulin-like (ShCML44) stress-responsive gene from Solanum habrochaites enhances tolerance to multiple abiotic stresses. Sci Rep. 6:31772.

Nelson MR, Thulin E, Fagan PA, Fourn S, Chazin WJ. 2002. The EF-hand domain: a globally cooperative structural unit. Protein Sci. 11(2):198–205.

Opitz N, Paschold A, Marcon C, Malik WA, Lanz C, Piepho HP, Hochholdinger F. 2014. Transcriptomic complexity in young maize primary roots in response to low water potentials. BMC Genomics. 15(1):741.

Panchy N, Lehtil-Shiu M, Shiu SH. 2016. Evolution of gene duplication in Plants. Plant Sci. 171(4):229–236.

Pereraon V, Aldon D, Galaud JP, Ranty B. 2011. Calmodulin and calmodulin-like proteins in plant calcium signaling. Biochimie. 93(12):2084–2093.

Scholz SS, Reichelt M, Vadassey J, Mithöfer A. 2015. Calmodulin-like protein CML37 is a positive regulator of ABA during drought stress. Plant Sci Rep. 10(8):7474.

Shukla S, Zhao C, Shukla D. 2019. Dewatering controls plant hormone perception and initiation of drought resistance signaling. Structure. 27(4):692–702.e3.

Sun Q, Yu S, Guo Z. 2020. Calmodulin-like (CML) gene family in Medicago truncatula: genome-wide identification, characterization and expression analysis. Int J Mol Sci. 21(19):1742.

Vandelle E, Vannozzi A, Dong W, Danzi D, Digby AM, Dal Santo S, Astegno A. 2018. Identification, characterization, and expression analysis of calmodulin and calmodulin-like genes in grapevine (Vitis vinifera) reveal likely roles in stress responses. Plant Physiol Biochem. 129:221–237.

Walley JW, Sartor RC, Shen Z, Schmitz RJ, Wu KJ, Urich MA, Nery JR, Smith LG, Schnable JC, Ecker JR, Briggs SP. 2016. Integration of
omic networks in a developmental atlas of maize. Science. 353 (6301):814–818.
Wang SS, Diao WZ, Yang X, Qiao Z, Wang M, Acharya BR, Zhang W. 2015. Arabidopsis thaliana CML25 mediates the Ca(2+) regulation of K+ transmembrane trafficking during pollen germination and tube elongation. Plant Cell Environ. 38(11):2372–2386.
Wang X, Zhu B, Jiang Z, Wang S. 2019. Calcium-mediation of jasmonate biosynthesis and signaling in plants. Plant Sci. 287:110192.
Wu X, Qiao Z, Liu H, Acharya BR, Li C, Zhang W. 2017. CML20, an Arabidopsis calmodulin-like protein, negatively regulates guard cell ABA signaling and drought stress tolerance. Front Plant Sci. 8:824.
Xu GY, Rocha PS, Wang ML, Xu ML, Cui YC, Li LY, Zhu YX, Xia X. 2011. A novel rice calmodulin-like gene, OsMSR2, enhances drought and salt tolerance and increases ABA sensitivity in Arabidopsis. Planta. 234(1):47–59.
Yang X, Wang SS, Wang M, Qiao Z, Bao CC, Zhang W. 2014. 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. 86 (3):225–236.
Yue R, Lu C, Sun T, Peng T, Han X, Qi J, Yan S, Tie S. 2015. Identification and expression profiling analysis of calmodulin-binding transcription activator genes in maize (Zea mays L.) under abiotic and biotic stresses. Front Plant Sci. 6:576.
Zaidi PH, Seetharam K, Krishna G, Krishnamurthy L, Gajanan S, Babu R, Zerka M, Vinayan MT, Vivek BS, Yadav RS. 2016. Genomic regions associated with root traits under drought stress in tropical maize (Zea mays L.). PLoS One. 11(10):e0164340.
Zeng H, Zhang Y, Zhang X, Pi E, Zhu Y. 2017. Analysis of EF-hand proteins in soybean genome suggests their potential roles in environmental and nutritional stress signaling. Front Plant Sci. 8:877.
Zhao F, Li G, Hu P, Zhao X, Li L, Wei W, Feng J, Zhou H. 2018. Identification of basic/helix-loop-helix transcription factors reveals candidate genes involved in anthocyanin biosynthesis from the strawberry white-flesh mutant. Sci Rep. 8(1):2721.
Zhu JK. 2016. Abiotic stress signaling and responses in plants. Cell. 167 (2):313–324.
Zhu X, Dunand C, Snedden W, Galaud JP. 2015. Cam and CML emergence in the Green lineage. Trends Plant Sci. 20(8):483–489.
Zhu X, Robe E, Jomat L, Aldon D, Mazars C, Galaud JP. 2017. CML8, an Arabidopsis calmodulin-like protein, plays a role in Pseudomonas syringae plant immunity. Plant Cell Physiol. 58(2):307–319.