



Article

Calmodulin-Like (CML) Gene Family in *Medicago truncatula*: Genome-Wide Identification, Characterization and Expression Analysis

Qiguo Sun, Shuhan Yu and Zhenfei Guo *

College of Grassland Science, Nanjing Agricultural University, Nanjing 210095, China; 2017220007@njau.edu.cn (Q.S.); 2018220001@njau.edu.cn (S.Y.)

* Correspondence: zfguo@njau.edu.cn; Tel.: +86-25-84396101

Received: 31 August 2020; Accepted: 25 September 2020; Published: 27 September 2020



Abstract: Calcium is an important second messenger in mediating adaptation responses of plants to abiotic and biotic stresses. Calmodulin-like (CML) protein is an important calcium-signaling protein that can sense and decode Ca^{2+} signal in plants. *Medicago truncatula* is a model legume plant; however, investigations of MtCML proteins are limited. Using genome analysis and BLAST database searches, fifty MtCML proteins that possess EF-hand motifs were identified. Phylogenetic analysis showed that CML homologs between *M. truncatula*, *Arabidopsis thaliana* and *Oryza sativa* shared close relationships. Gene structure analysis revealed that these MtCML genes contained one to four conserved EF-hand motifs. All MtCMLs are localized to eight chromosomes and underwent gene duplication. In addition, MtCML genes were differentially expressed in different tissues of *M. truncatula*. *Cis*-acting elements in promoter region and expression analysis revealed the potential response of MtCML protein to abiotic stress and hormones. The results provide a basis of further functional research on the MtCML gene family and facilitate their potential use for applications in the genetic improvement on *M. truncatula* in drought, cold and salt stress environments.

Keywords: CML; *Medicago truncatula*; expression profiling; promoter *cis*-acting element; abiotic stress

1. Introduction

Calcium is a universal second messenger in mediating adaptation responses of plants to abiotic and biotic stresses [1]. Diverse stimuli such as plant hormones, low temperature, drought, salt, and pathogens induce rapid and transient changes in cellular Ca^{2+} concentration [2], which are sensed and decoded by calcium-binding proteins (CBP) to activate downstream reactions [3]. The CBPs include calmodulins (CaMs), CaM-like proteins (CMLs), Ca^{2+} -dependent protein kinases (CPKs/CDPKs) and calcineurin B-like proteins (CBLs) in plants [4–6]. A typical CaM contains four EF-hand motifs [7], while an EF-hand motif is composed of two helices, the E helix and the F helix, flanking a Ca^{2+} -binding loop in a structure that resembles a hand [8]. CMLs are a unique class of EF-hand proteins and specifically existed in plants. The CML gene family has been characterized in some plant species, such as *Arabidopsis* [9], rice [10], grapevine (*Vitis amurensis*) [11], Chinese cabbage (*Brassica rapa*) [12] and tomato (*Solanum lycopersicum*) [13].

CMLs regulate downstream targets in response to various stimuli-induced Ca^{2+} fluctuations and signal transduction [14]. In *Arabidopsis*, CML15 and CML16 show changes in electrophoretic mobility in the presence of Ca^{2+} , which confirms their ability to bind Ca^{2+} [15,16]. The roles of CMLs in plant development and stress responses were investigated [17–19]. CML24 regulates root mechanoresponses, pollen tube growth and flowering in *Arabidopsis* [20–23]. CML24 can respond circadian oscillations of cytosolic-free calcium and regulate the circadian clock [24]. The other

CMLs, such as CML12, CML25, CML38, CML39 and CML43, are involved in plant growth and development [25–29]. CML8, CML41, CML42, CML46 and CML47 are involved in plant resistance to bacterium pathogens [30–33], while CML9, CML24, CML37, CML38, and CML39 transcripts are induced by salt or/and drought stress [2,23,34,35]. CML20 is a negative regulator, while CML24 and CML37 are positive regulators in ABA-signaling during drought stress [23,34,36]. OsCML16, a direct target of OsERF48, appears to transduce OsERF48 actions to downstream target genes that together confer the acquired root phenotype and drought tolerance in rice [37]. GsCML27 is a positive regulator of plant tolerance to bicarbonate stress, but a negative regulator of salt or osmotic stresses during early growth stages in *Glycine soja* [38]. CML proteins play crucial roles in diverse physiological processes.

M. truncatula is an important model leguminous plant [39]. MtCML40 is a negative regulator of salt tolerance in *M. truncatula* by downregulating *MtHKTs* expression and leading to Na⁺ toxicity [40]. However, the other MtCMLs have not been investigated. The objective of this study was to perform a genome-wide analysis of CMLs in *M. truncatula* including gene structure, chromosomal location, duplication, EF-hand motif organization and expression characteristics. The results provide a comprehensive understanding of the CML gene family in *M. truncatula*.

2. Results

2.1. Identification of CML Members in *M. truncatula*

A genome-wide search for CML genes was performed using the BLASTP program based on the completed genome sequence of *M. truncatula*, using *Arabidopsis* and rice CML genes as the query sequences. Fifty MtCML genes were obtained and their deduced peptides were subjected to domain analysis using Pfam and SMART databases for further confirmation. A total of 50 members were identified, and they were named as MtCML1 to MtCML50 (Table 1). The amino acid sequence of MtCMLs was further analyzed. MtCMLs had an average of approximately 167 amino acids in length, ranging from 65 amino acids in MtCML28 to 266 amino acids in MtCML11. The predicted molecular weight varied from 7.37 kDa to 29.98 kDa, and the theoretical isoelectric point (pI) varied from 4.01 to 9.01 (Table 1). The predicted grand average of hydropathicity (GRAVY) of all MtCMLs was negative, indicating that MtCMLs are hydrophilic proteins (Table 1).

Table 1. Information on MtCML proteins.

Name	Locus ID	CDS (bp)	A.A.	Mw (kDa)	pI	GRAVY
MtCML1	Medtr1g019600.1	303	101	11.57	8.73	−0.412
MtCML2	Medtr1g019610.1	294	98	11.29	7.75	−0.744
MtCML3	Medtr1g019640.1	288	96	10.91	9.01	−0.747
MtCML4	Medtr1g019660.1	288	96	10.88	6.27	−0.589
MtCML5	Medtr1g030440.1	504	168	19.17	4.26	−0.137
MtCML6	Medtr1g032070.1	597	199	21.91	4.36	−0.532
MtCML7	Medtr1g041285.1	453	151	17.03	4.01	−0.401
MtCML8	Medtr1g046950.1	429	143	16.04	4.54	−0.675
MtCML9	Medtr1g047100.1	426	142	15.75	4.43	−0.537
MtCML10	Medtr1g076650.1	453	151	17.16	4.07	−0.498
MtCML11	Medtr2g086560.1	798	266	29.98	6.59	−0.703
MtCML12	Medtr2g098890.1	636	212	24.24	4.77	−0.319
MtCML13	Medtr3g067610.1	417	139	15.71	4.99	−0.862
MtCML14	Medtr3g088655.1	495	160	18.47	4.35	−0.481
MtCML15	Medtr3g089070.1	423	141	16.03	4.55	−0.474
MtCML16	Medtr3g089090.1	423	140	15.90	4.55	−0.491
MtCML17	Medtr3g109320.1	540	180	20.65	4.58	−0.475
MtCML18	Medtr4g067270.1	453	151	17.08	4.37	−0.546
MtCML19	Medtr4g082050.1	543	144	16.32	4.38	−0.175

Table 1. Cont.

Name	Locus ID	CDS (bp)	A.A.	Mw (KDa)	pI	GRAVY
MtCML20	Medtr4g086260.1	591	197	22.73	8.42	-0.704
MtCML21	Medtr4g103630.1	423	141	16.00	4.43	-0.494
MtCML22	Medtr4g112460.1	510	170	18.74	4.5	-0.087
MtCML23	Medtr4g115170.1	681	226	25.26	4.46	-0.219
MtCML24	Medtr4g127560.1	600	200	23.04	4.48	-0.368
MtCML25	Medtr5g008695.1	348	115	13.15	4.38	-0.63
MtCML26	Medtr5g008705.1	393	131	14.90	4.68	-0.706
MtCML27	Medtr5g011850.1	444	148	16.59	4.64	-0.418
MtCML28	Medtr5g011920.1	195	65	7.37	6.71	-0.17
MtCML29	Medtr5g017510.1	570	190	21.24	6.63	-0.584
MtCML30	Medtr5g017550.1	567	189	21.16	6.64	-0.661
MtCML31	Medtr5g017560.1	570	190	21.24	6.63	-0.584
MtCML32	Medtr5g025690.1	603	201	23.26	4.34	-0.325
MtCML33	Medtr5g079340.1	420	140	15.78	4.56	-0.689
MtCML34	Medtr6g007613.1	513	171	19.80	4.86	-0.88
MtCML35	Medtr6g023460.1	429	143	15.95	4.37	-0.32
MtCML36	Medtr6g079570.1	525	175	19.31	4.35	-0.584
MtCML37	Medtr7g011010.1	609	203	23.61	4.46	-0.307
MtCML38	Medtr7g074020.1	516	172	19.77	4.84	-0.961
MtCML39	Medtr7g074240.1	537	179	20.08	4.45	-0.357
MtCML40	Medtr7g075040.1	588	196	22.01	4.71	-0.352
MtCML41	Medtr7g089760.1	459	153	16.97	4.16	-0.472
MtCML42	Medtr7g090450.1	651	217	23.86	4.67	-0.478
MtCML43	Medtr7g451050.1	717	239	27.95	5.08	-0.455
MtCML44	Medtr8g036075.1	597	199	22.55	5.32	-0.36
MtCML45	Medtr8g066630.1	495	165	18.40	4.6	-0.373
MtCML46	Medtr8g069915.1	510	170	18.77	4.26	-0.242
MtCML47	Medtr8g070510.1	684	228	26.11	4.65	-0.442
MtCML48	Medtr8g078270.1	567	189	21.51	5.01	-0.66
MtCML49	Medtr8g105230.1	573	191	20.77	4.35	-0.523
MtCML50	Medtr8g107110.1	498	166	18.22	4.31	-0.553

Note: A.A.—number of amino acids sequence; pI—theoretical isoelectric point of proteins; MW—theoretical molecular weight of proteins; GRAVY—grand average of hydropathicity.

2.2. Phylogenetic Relationship of CMLs among *M. truncatula*, *Arabidopsis* and Rice

To evaluate the evolutionary relationship, all CML members from *M. truncatula* (50), *Arabidopsis* (50) and rice (32) were aligned using the maximum-likelihood (ML) method to generate an unrooted phylogenetic tree (Figure 1). The sequences of MtCML proteins are shown in Table S1. CML proteins in these species could be clustered into eight groups (Groups 1–8). The results suggest existence of a common ancestor before the divergence of monocots and dicots. More members were grouped in Group 3, 6, 7 and 8 than in others. Group 3 contained *Arabidopsis* and *M. truncatula* CMLs members, but not rice CMLs (Figure 1), indicating that there are differences in the evolution of CML proteins between monocots and dicots. In addition, most of the MtCML showed homologous to those in *Arabidopsis* and rice (Figure 1). The results indicated that CMLs are conserved among plant species.

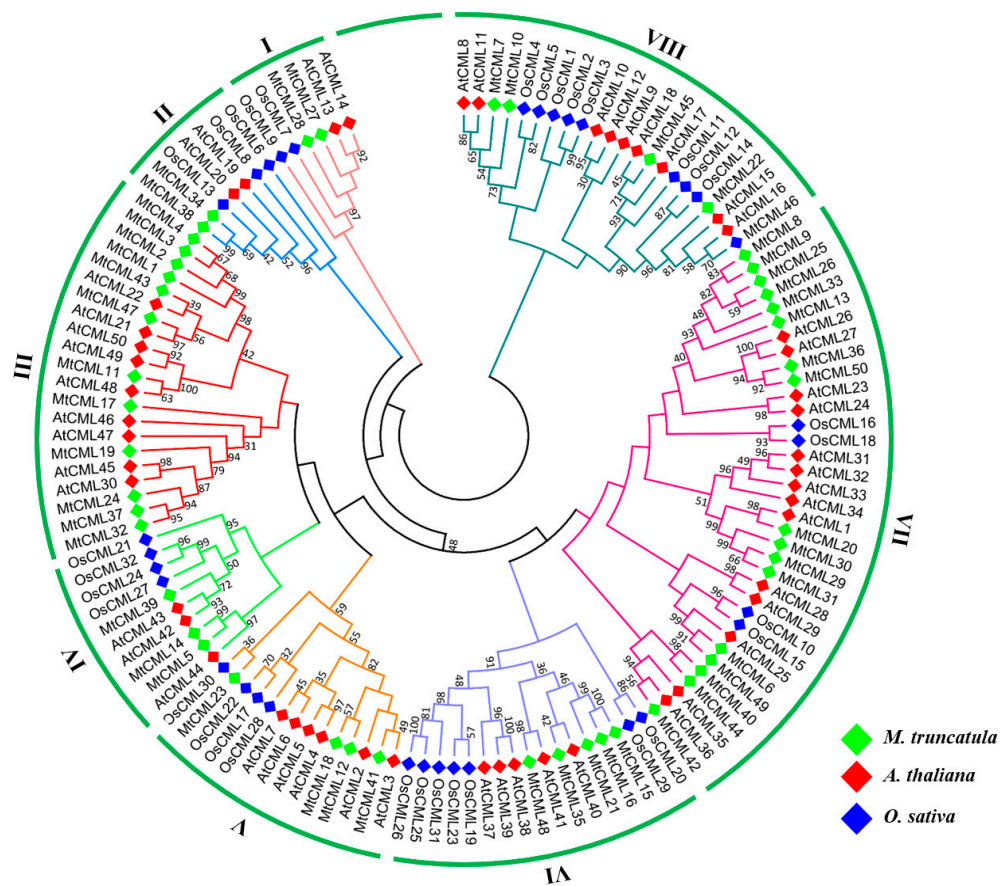


Figure 1. Phylogenetic tree of calmodulin-like (CML) proteins in *Medicago truncatula*, *Arabidopsis thaliana* and *Oryza sativa*. Tree constructed with 1000 bootstrap replications. CMLs from *M. truncatula*, *A. thaliana* and *O. sativa* distinguished using green, red and blue color, respectively.

2.3. Gene Structure and Domain Architectures

MtCMLs structure was analyzed based on the arrangement of exon/intron. No intron was found in 40 members of *MtCMLs*, while one to six introns were found in the others. One intron was found in three *MtCMLs* (*MtCML28*, *MtCML39* and *MtCML46*); three introns in four *MtCMLs* (*MtCML7*, *MtCML10*, *MtCML43* and *MtCML47*), four in *MtCML11* and six in *MtCML34* and *MtCML38* (Figure 2A).

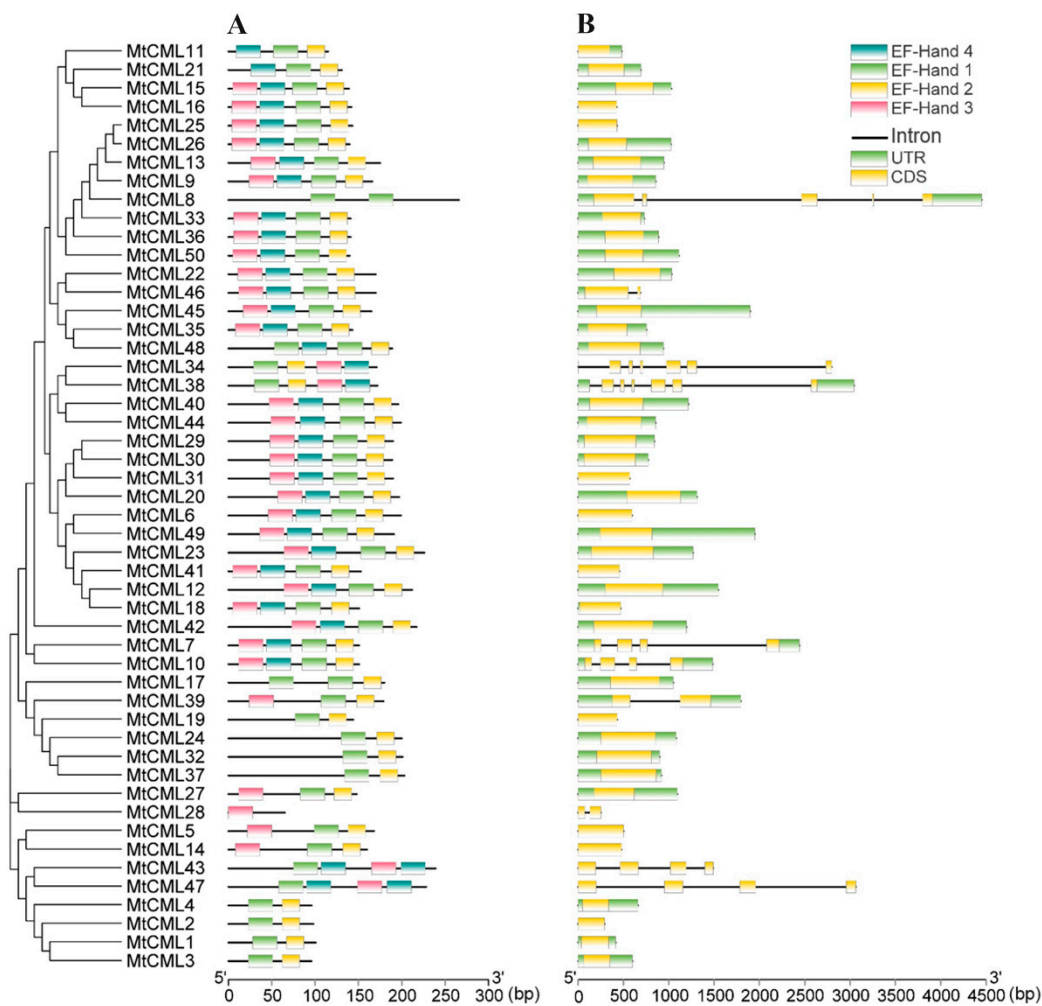


Figure 2. Characterization of *MtCML* genes and MtCML proteins. (A) EF-hand motifs; (B) exon–intron structure distribution.

EF-hand motifs are the most prominent structural feature in CML proteins. Most of MtCML members (33) contained four conserved EF-hand motifs, and seven members contained three EF-hand motifs. Two EF-hand motifs were present in nine MtCML members (MtCML1, MtCML2, MtCML3, MtCML4, MtCML11, MtCML19, MtCML24, MtCML32 and MtCML27), while one EF-hand motif in MtCML28 (Figure 2B).

2.4. Chromosomal Location and Synteny Analysis of *MtCML* Genes

MtCMLs were mapped onto the chromosomes against the *M. truncatula* genome database to examine their chromosomal distribution. *MtCMLs* were located on chromosome 1 to 8. Ten *MtCMLs* (MtCML1, MtCML2, MtCML3, MtCML4, MtCML5, MtCML6, MtCML7, MtCML8, MtCML9 and MtCML10) were located on chromosome 1, while nine (MtCML25, MtCML26, MtCML27, MtCML28, MtCML29, MtCML30, MtCML31, MtCML32 and MtCML33) on chromosome five. There were seven *MtCMLs* locating on chromosomes four, seven and eight, respectively. In addition, five *MtCMLs* (MtCML13, MtCML14, MtCML15, MtCML16 and MtCML17) were located on chromosome three, three (MtCML34, MtCML35 and MtCML36) on chromosomes six and two (MtCML11 and MtCML12) on chromosome two (Figure 3A). The results indicated that *MtCMLs* were randomly scattered on different chromosomes.

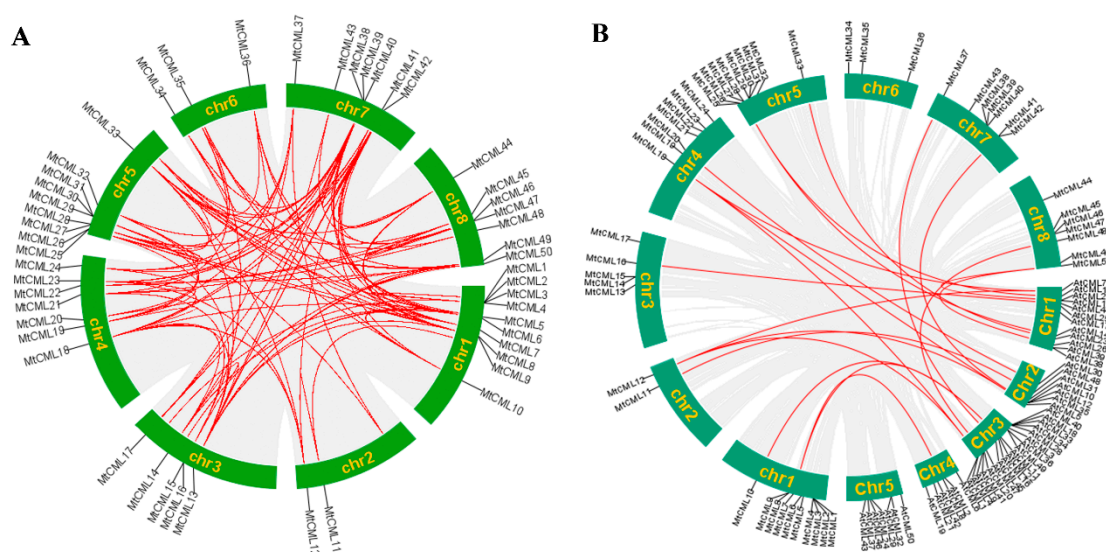


Figure 3. Chromosomal distribution and synteny analysis of CML genes in the genomes of *Medicago truncatula* and *Arabidopsis thaliana*. (A) Paralogous MtCML genes mapped onto *M. truncatula* chromosomes; (B) Orthologous CML genes mapped onto *M. truncatula* (chromosomes 1–8) and *A. thaliana* (chromosomes 1–5). Red lines indicate duplicated MtCML gene pairs.

Collinearity diagrams among MtCMLs were further analyzed. The results showed that all MtCML genes underwent gene duplication in *M. truncatula* genome (Figure 3A, Table S7). Most MtCML genes were found with 1–3 paralogous genes in *M. truncatula*. Five MtCML genes (*MtCML5*, *MtCML6*, *MtCML8*, *MtCML12* and *MtCML20*) had four homologous genes in *M. truncatula*. In addition, a synteny analysis of CML genes among *M. truncatula* and *A. thaliana* was performed. Twenty pairs of orthologous CMLs were identified between *M. truncatula* and *A. thaliana* (Figure 3B, Table S7). Three genes (*MtCML7*, *MtCML27* and *MtCML50*) had two homologous genes in *A. thaliana*, while two genes (*MtCML12* and *MtCML18*) had three orthologous genes in *A. thaliana*. The percentage of identity between pairs of paralogous MtCML proteins ranged from 21.69% to 100% in *M. truncatula* (Table S7). Among *M. truncatula* and *A. thaliana*, the identity between pairs of orthologous ranged 28.06% to 87.16%. Some high percentages of identity between MtCMLs and AtCMLs suggest that MtCML protein sequences and functions could be highly conserved. These results indicated that MtCML genes may be generated by gene duplication, which may be associated with conserved amino acid sequence in MtCML.

2.5. Spatial and Temporal Expression Profiles of MtCMLs

The spatial and temporal expression profiles of MtCMLs were examined based on the microarray data from *M. truncatula* Gene Expression Atlas (MtGEA, <https://mtgea.noble.org/v3/>). Twenty-nine MtCMLs have corresponding probe sets in the dataset (Table S2). *MtCML13*, *MtCML27*, *MtCML36*, *MtCML38* and *MtCML42* exhibited relatively high expression in all tissues (flowers, leaves, petioles, pods, stems, roots and seeds) (Figure 4A), indicating that their functions may be extensive. *MtCML7*, *MtCML14*, *MtCML17* and *MtCML22* were preferentially expressed in roots, and *MtCML47* was only expressed in mature seeds, while *MtCML20* and *MtCML30* were expressed in leaves. Some MtCMLs (*MtCML6*, *MtCML13*, *MtCML25*, *MtCML28* and *MtCML30*) were highly expressed in floral organs (Figure 4A). On the other hand, relative expression of *MtCML7*, *MtCML14*, *MtCML17*, *MtCML20*, *MtCML22* and *MtCML47* was examined using qRT-PCR (Figure 4B–G). *MtCML7* was mostly expressed in roots, stems and leaves, but not in seeds (Figure 4B); *MtCML14* was highly expressed in leaves and its transcript could be detected in roots, stems, flowers and seeds (Figure 4C). *MtCML17* was highly expressed in roots, and its transcript could be detected in stems, flowers and pods (Figure 4D). *MtCML20* was major expressed in leaves and flowers as well as in stems, but not in roots and pods (Figure 4E),

MtCML22 was only highly expressed in roots (Figure 4E). *MtCML47* was expressed in all detected organs with highest transcript in leaves (Figure 4F). The expression patterns of *MtCML7*, *MtCML17*, *MtCML20*, *MtCML22* and *MtCML47* were consistent with that in microarray data. The different spatial and temporal expression patterns of the *MtCML* genes suggest their functional diversity in *Medicago* development.

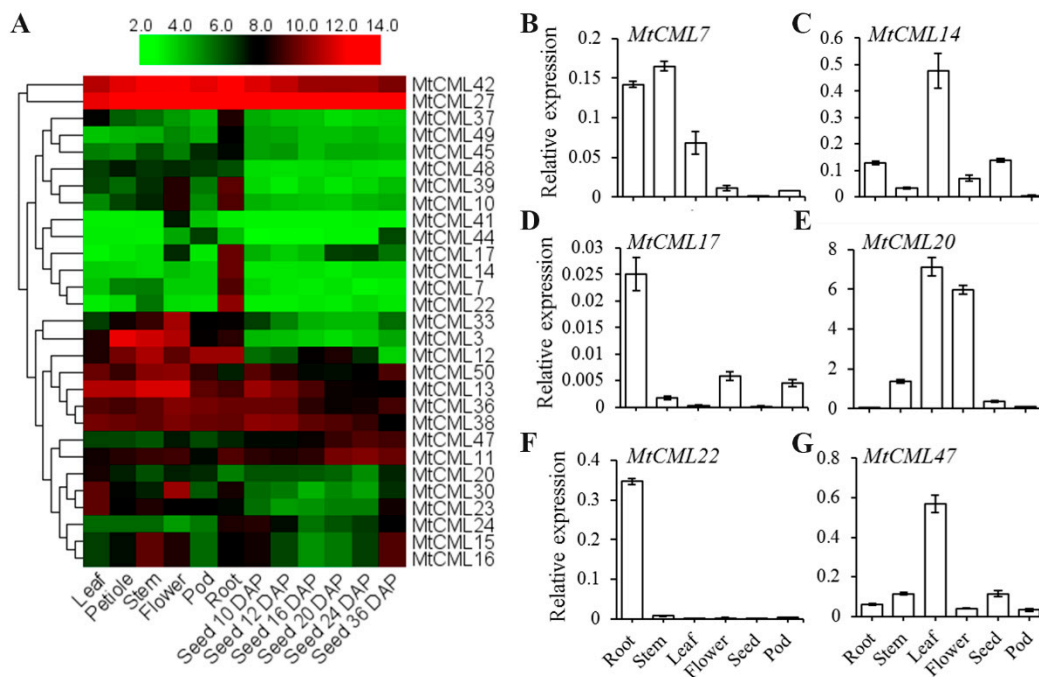


Figure 4. Expression analysis of *MtCML* genes in different organs. (A) Expression patterns of *MtCML* genes in leaves, petioles, stems, flowers, pods, roots and seeds. The expression levels of *MtCML* genes are shown as the log₂-based fluorescence intensity values from the microarray data (MtGEA, <https://mtgea.noble.org/v3/>). DAP, days after pollination; (B–G) relative expression of (B) *MtCML7*; (C) *MtCML14*; (D) *MtCML17*; (E) *MtCML20*; (F) *MtCML22*; (G) *MtCML47* in roots, stems, leaves, flowers, seeds and pods of *Medicago truncatula*. Expression data obtained via qRT-PCR. *MtActin* used as an internal control in the qRT-PCR experiments.

2.6. Analysis of cis-Acting Element in the Promoter Region of *MtCML* Genes

To analyze the potential function of *MtCML* genes, a 1000-bp sequence upstream start codon in the promoter region of *MtCMLs* was analyzed using the PlantCARE. The major *cis*-acting elements related to stress and phytohormone responses are shown in Table S5. ABA (ABRE), MeJA (CGTGA-motif) and auxin (AuxRR-core) response elements were enriched in the promoter of *MtCMLs*, while salicylic acid (TCA-motif), gibberellin (GARE-motif), circadian, drought (MBS) and cold (LTR) response elements were also found in the promoters (Figure 5). Seven *MtCMLs* (*MtCML8*, *MtCML20*, *MtCML28*, *MtCML33*, *MtCML35*, *MtCML37* and *MtCML40*) have drought response element, and six *MtCMLs* (*MtCML2*, *MtCML6*, *MtCML16*, *MtCML19*, *MtCML33* and *MtCML47*) have cold response element in the promoter (Figure 5). The results indicated that *MtCMLs* may respond to plant hormones and abiotic stresses. *MtActin* was used as an internal control to ascertain if the different *cis*-elements found in the promoters of the *MtCML* genes were or not substantially enriched [41]. Three *cis*-acting elements including two gibberellin responsive elements (GARE-motif and *P*-box), three salicylic acid responsiveness elements (TCA-element) and one light responsiveness (Box 4) were observed in the promoter region of *MtActin* (Table S5), while LTR, ABRE, CGTGA-motif, MBS and AuxRR-core elements were enriched in the *MtCML* promoters regions (Table S5).

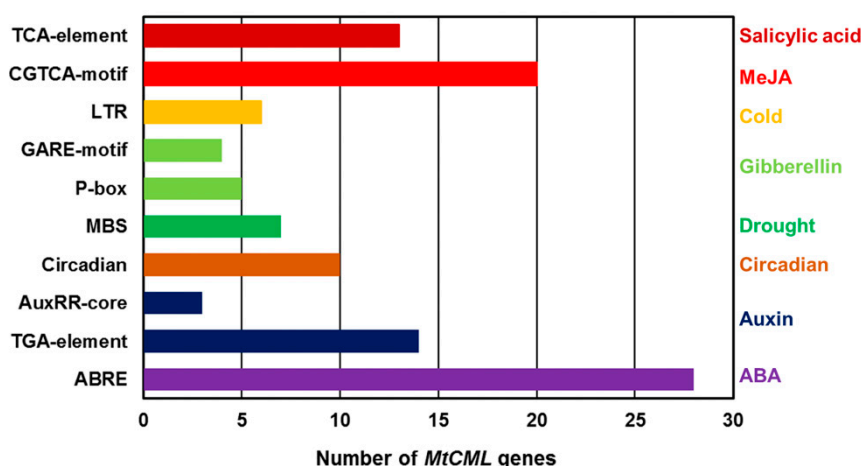


Figure 5. Number of *MtCML* genes containing various *cis*-acting elements. ABRE, ABA-responsive element. LTR—*cis*-acting element involved in low-temperature response; CGTCA-motif—*cis*-acting element involved in MeJA response; TCA-element—*cis*-acting element involved in SA response; GARE-motif and P-box—*cis*-acting element involved in GA response; MBS—*cis*-acting element involved in drought response; circadian—*cis*-acting element involved in biologic rhythms; AuxRR-core and TGA-element—*cis*-acting elements involved in auxin response.

2.7. Expression Profiles of *MtCMLs* in Response to Salt, Drought and Cold

Gene expression profiles of *MtCMLs* in different tissues under salt, drought and cold stresses were obtained from *M. truncatula* Gene Expression Atlas (MtGEA, <https://mtgea.noble.org/v3/>). There are 29 *MtCML* genes that have corresponding probe sets in the dataset [42]. *MtCMLs* expression patterns were changed in response to salt and drought stress (Table S3). *MtCML24* and *MtCML50* were significantly upregulated after six hours of salt stress, whereas *MtCML17* was downregulated (Figure 6A). In the hydroponic treatment experiment, *MtCML10*, *MtCML15*, *MtCML16* and *MtCML50* transcripts were upregulated after one hour of salt stress and downregulated at 10 h (Figure 6B). Most of *MtCML* transcripts were unaltered in shoot during drought treatment except for *MtCML33* and *MtCML47*, whose expression was increased significantly (Figure 6C). On the other hand, *MtCML15*, *MtCML16*, *MtCML23*, *MtCML47* and *MtCML50* transcripts in roots were induced by drought treatment, followed by decrease after rewatering, while *MtCML7*, *MtCML14*, *MtCML22*, *MtCML37*, *MtCML39*, *MtCML45* and *MtCML49* transcripts were significantly reduced after drought treatment followed by increase after rewatering (Figure 6D). The results indicated that *MtCML* may participate in drought and salt stress responses.

Expression of six *MtCML* genes that have LTR *cis*-acting element in the promoter regions in response to cold was analyzed (Table S4). *MtCML16* and *MtCML33* transcript levels were induced after six hours of cold treatment, while *MtCML2*, *MtCML6* and *MtCML19* transcripts were significantly reduced (Figure 7). *MtCML47* showed significant downregulation after one hour of cold treatment. The results indicated that *MtCML16*, *MtCML33*, *MtCML2*, *MtCML6*, *MtCML19* and *MtCML47* may participate in cold adaptation in *M. truncatula*.

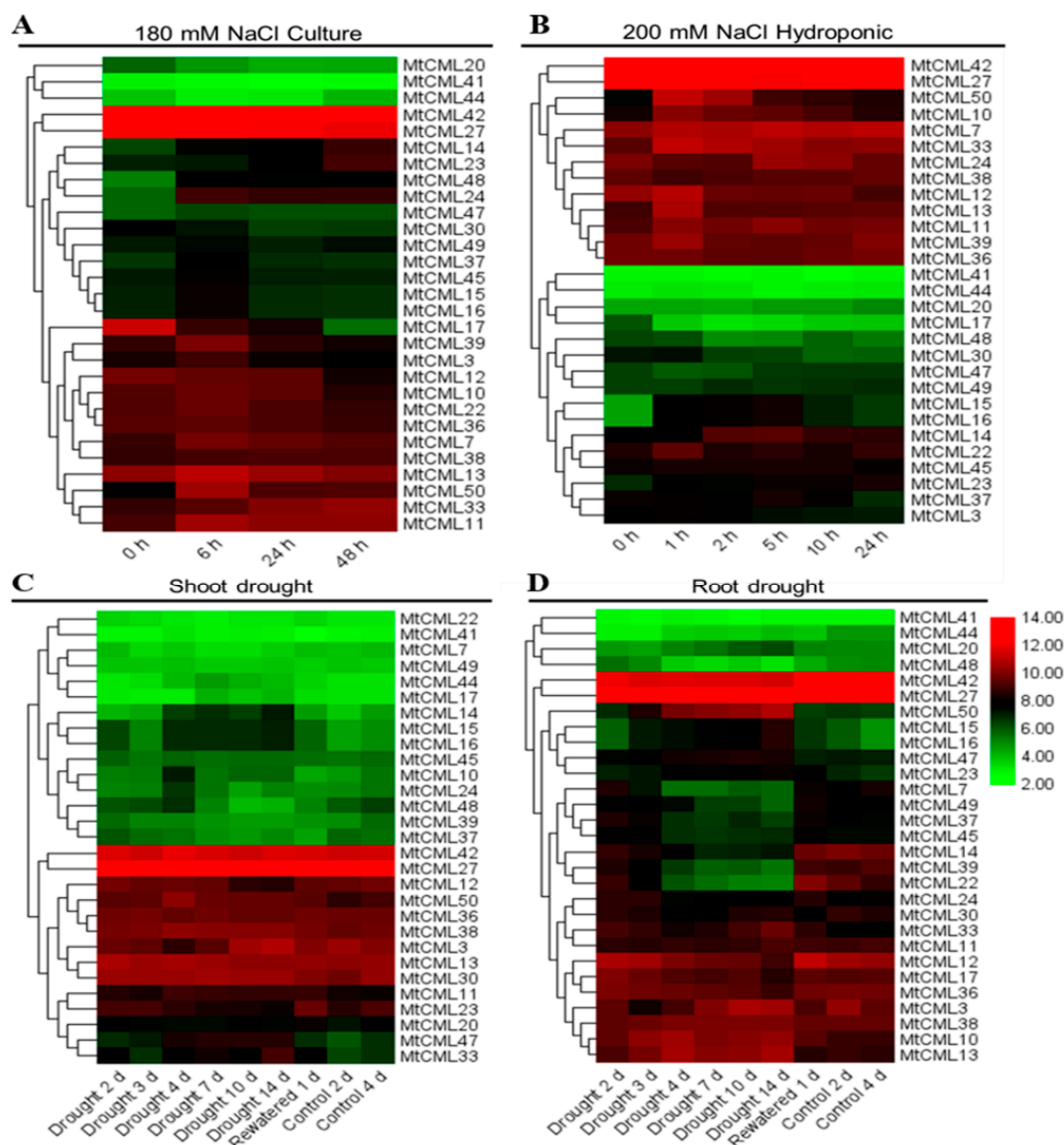


Figure 6. Transcript profile of *MtCML* genes in response to salt and drought stresses. Genome-wide microarray data of *M. truncatula* in different tissues at various developmental stages and the response to drought and salt were retrieved from *M. truncatula* Gene Expression Atlas (MtGEA, <https://mtgea.noble.org/v3/>). (A) Two-day-old seedlings were placed on half strength of MS medium containing 180 mM NaCl for 0, 6, 24 and 48 h, (B) Two-week-old seedlings were treated in a nutrient solution containing 200 mM NaCl for 1, 2, 5, 10 and 24 h as hydroponic treatment, while those growing in the nutrient solution as control. C and D, 24-day-old seedlings growing in soil were stopped watering for drought treatment for 14 d before rewatering, followed by sampling shoot and roots, respectively for analysis of gene expression. The expression levels of the *MtCML* genes are shown as the log₂-based fluorescence intensity values from the microarray data (MtGEA, <https://mtgea.noble.org/v3/>).

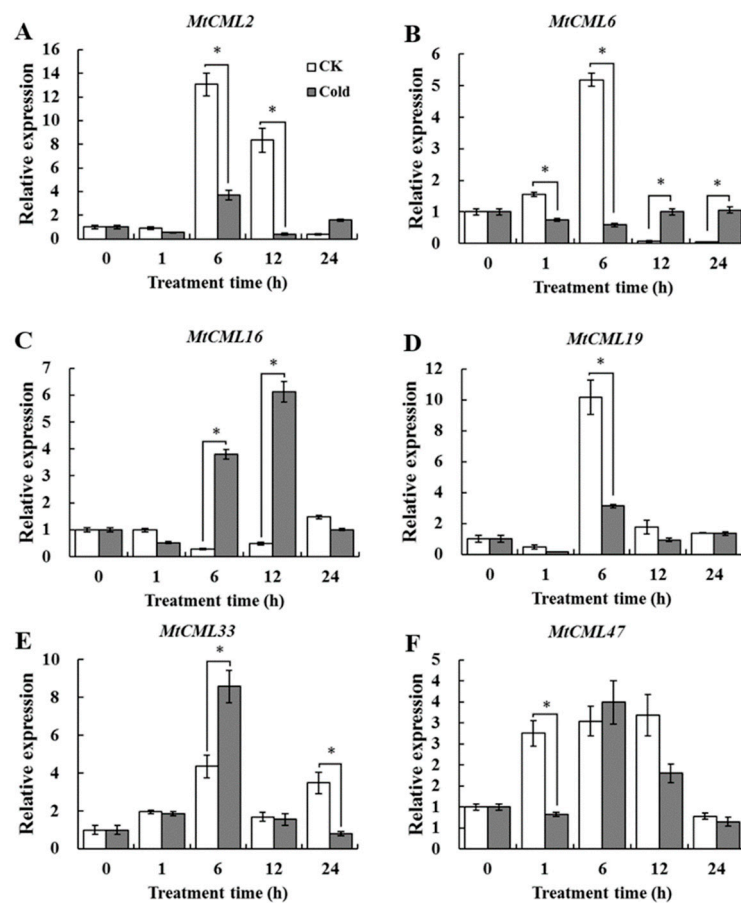


Figure 7. Relative expression of (A) *MtCML2*, (B) *MtCML6*, (C) *MtCML16*, (D) *MtCML19*, (E) *MtCML33* and (F) *MtCML47* in response to cold treatment. Mean values and standard errors of three independent experiments presented. * indicate significant difference between cold treatment and control at $p < 0.05$.

3. Discussion

Camodulin-like proteins play a key role in signal transduction during plant growth and development [3]. A total of 50 *CML* genes were identified in *M. truncatula* in this study, which was same to that in *Arabidopsis* (50 *CML* members) [9], but different from that in rice (32 *CML* genes) [10], grapevine (68 *CML* genes), Chinese cabbage (79 *CML* genes) [12] and tomato (52 *CML* genes) [13]. *MtCMLs* show extensive variations in gene length, predicted protein size and pI. The varying protein size and pI and hydrophilic property of *MtCMLs* are consistent with that in *CMLs* of Chinese cabbage [12,43]. The function of *CML* members are dependent upon their amino acids sequences, number of EF-hand motifs that are implicated in the Ca^{2+} -binding properties and their responses to plant hormones and environmental stresses [8,44]. The number of EF-hand motifs varies among plant species. *Arabidopsis* *CML* proteins typically possess two to six EF-hand motifs [9], but one to four conserved EF-hand motifs were found in *MtCMLs*. The diversity of *MtCMLs* in structure may result in functional diversity. The intron–exon structural analysis showed that most of *MtCML* genes have no intron. This case was consistent with *AtCMLs*, *OsCMLs* and *BrCMLs* [9,10,12].

MtCMLs were classified into eight groups, which is likely similar to those in other plant species [9,12,13]. Phylogenetic analysis of *CML* proteins revealed that many *MtCMLs* were homologous to those in *Arabidopsis* and rice. In consistence, most of *BrCMLs* from *Brassica rapa* were also closely related to their corresponding homologs in *Arabidopsis* and rice [12]. These results suggest that *CMLs* are conserved among plant species. Gene duplication events are important in the rapid expansion and evolution of gene families [45]. In our study, chromosomal distribution analysis revealed that all *MtCML* genes were randomly scattered on different chromosomes. Homologous gene pairs were

found in almost all *MtCMLs*. There are many homologous *CML* genes between the chromosomes in *M. truncatula* and *Arabidopsis*. Whole-genome duplication (WDG) in legumes played a major role in shaping the genome and contributed the raw material for the evolution of nodulation [39,46]; *M. truncatula* has undergone high rates of local gene duplication and share an ancient round of gene duplications from other legume species [39,47]. Therefore, WGD and tandem duplication probably participated in driving *MtCML* genes evolution.

The tissue-specific expression patterns of *MtCML* genes are presumed to be associated with their potential biologic roles. Four *MtCMLs* (*MtCML6*, *MtCML13*, *MtCML25* and *MtCML28*) were highly expressed in floral organs, indicating that they are associated with flowering regulation. This can be supported by that *AtCML25*, the homolog of *MtCML25*, is involved in pollen germination and pollen tube elongation via regulating Ca^{2+} and K^{+} transmembrane trafficking in pollen grains and pollen tubes in *Arabidopsis* [25]. *MtCML17* and *MtCML22* were highly expressed in roots, indicating that they are associated with roots in *M. truncatula*. However, qRT-PCR showed that *MtCML14* and *MtCML47* were mostly expressed in leaves and seeds rather than roots (Figure 4B–G). The expression difference between qRT-PCR and microarray data may result by the difference of plant growth stages.

The temporal, spatial, and cell type-specific expression of genes are associated with the regulatory elements in the promoter region [48]. Some promoter elements (LTR, ABRE, CGTGA-motif, MBS and AuxRR-core) were enriched multiple times in the promoter regions of *MtCML* genes, but were not found in *MtActin*. It suggested that *MtCML* genes can be involved in regulating multiple hormone responses and abiotic stresses. Previous studies have shown that some *CML* genes participated in the hormonal or abiotic stresses responses when specific *cis*-elements were found in their promoter regions, like *AtCML9* [49], *AtCML20* [36], *AtCML37* [34] and *MtCML40* [40]. This is consistent with our hypothesis. The expression of *CML* genes varies specifically in response to Ca^{2+} signaling and a variety of stress responses, especially abiotic stresses. In the present study, four *MtCML* genes (*MtCML14*, *MtCML17*, *MtCML30* and *MtCML50*) and nearly half of the *MtCML* genes showed extensive responses to salt and drought stresses, respectively. Similar to these results, studies have shown that *AtCML9* was induced under dehydration treatment [2]. Transcripts of *AtCML9*, *AtCML37*, *AtCML38*, *AtCML39* and *OsMSR2* (a rice *CML*) were up-regulated substantially following salt stress [2,35,50]. Although *MtCML40* is not found in the database, its expression was induced by salt treatment and negatively regulates salt tolerance in *M. truncatula* [40]. Six *MtCML* genes showed response to cold stress. *AtCML24* transcript is induced after cold treatment in *Arabidopsis* [23]. The tomato (*Solanum habrochaites*) *CML* gene, *ShCML44*, was found to improve the tomato tolerance in cold, drought and salinity stresses [51]. These previous studies have shown that *CML* can play a role in response to salt, drought and low temperature stresses, which is consistent with our qRT-PCR results. These results will help further explore the function of *MtCML* genes in *M. truncatula*.

4. Materials and Methods

4.1. Identification of *CML* Genes in *M. truncatula*

The *M. truncatula* Mt4.0v1 protein sequences were downloaded from Phytozome 12 (<https://phytozome.jgi.doe.gov/>). The reported *CML* gene sequences in *A. thaliana* were downloaded from the TAIR database (<https://www.arabidopsis.org/>) and used as a query to perform BLASTP searching. The NCBI database was used to search the potential *CML* genes in *M. truncatula*. In addition, the protein sequences of *OsCMLs* was downloaded from TIGR (<http://rice.plantbiology.msu.edu/>) and used as queries to search against the *M. truncatula* proteome. To differentiate CaM and *CML* genes which were homologs in *M. truncatula*, we follow the principle of the major character of EF-hand-containing proteins [13,43] and screen the candidate *CML* genes. All resulting non-redundant protein sequences were checked for the presence of the entire EF-hand domain by SMART (<http://smart.embl-heidelberg.de/>) and InterProScan (<http://www.ebi.ac.uk/interpro/>).

4.2. Analysis of Conserved Domain, Gene Structure and Characterization of MtCML Genes

The MtCML protein sequences were analyzed for physical and chemical characteristics, including the molecular weight (MW), theoretical point (pI) and grand average of hydropathicity (GRAVY), using the ProtParam tool of ExPASy (<http://web.expasy.org/protparam/>). The exon–intron structure analysis of MtCML genes was conducted using the TBtools (Toolbox for Biologists) program with default parameters. The conserved motifs were analyzed using the MEME tool (<http://meme-suite.org/tools/meme>), with the minimum width of motifs as 10, the maximum width of motifs as 40 and the other parameters as default values.

4.3. Phylogenetic Relationships of CML Proteins in *M. truncatula*, *Arabidopsis* and Rice

The multiple alignments of MtCML proteins were performed using ClustalX with default parameters. Phylogenetic analysis of CMLs in *M. truncatula*, *Arabidopsis* and rice was performed using MEGA X with the maximum-likelihood (ML) method 1000 bootstrap replicates [52].

4.4. Chromosomal Locations of MtCML Genes

The sequences of MtCML were used to retrieve their chromosomal locations in *M. truncatula* genome databases Phytozome 12 (<https://phytozome.jgi.doe.gov/>). TBtools software was used for analyze the chromosomal locations and homologous relationship of MtCML genes [53]. The Multiple Collinearity Scan toolkit (MCScanX) was used to analyze the gene duplication events [53].

4.5. Analysis of cis-Acting Elements of MtCML Genes

A 1000 bp of genomic DNA sequence upstream of the transcriptional start site in each MtCML was obtained from the Phytozome database (<https://phytozome.jgi.doe.gov/>), and the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [54] was used to identify the cis-acting elements in the promoter region of MtCMLs.

4.6. Analysis of Microarray Expression Profile

The genome-wide microarray data of *M. truncatula* in different tissues at various developmental stages and the response to drought and salt were retrieved from *M. truncatula* Gene Expression Atlas (MtGEA, <https://mtgea.noble.org/v3/>). The transcript data of MtCMLs were analyzed using TBtools software. The normalized expression data were used to generate heatmap using the TBtools software [53].

4.7. Analysis of Relative Expression of MtCMLs in Different Tissues and Response to Cold Using qRT-PCR

M. truncatula (Jemalong) A17 plants were grown in 15 cm-diameter plastic pots containing a mixture of peat and perlite (3:1, v/v) in a greenhouse with temperature ranging from 20 to 28 °C under natural light. Four-week-old plants were exposed to low temperature in a growth chamber at 4 °C for 24 h under 12 h light as cold treatment. Total RNA was extracted using RNAPrep pure Plant Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The cDNA was synthesized from 1 µg of total RNA, using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). MtCML genes transcript were analyzed using qRT-PCR in Thermal Cycler Dice Real Time System II (Takara, Japan) following the manufacturer's instructions. The PCR reaction mix was consisted of diluted cDNA as template, 200 nM forward and reverse primers and 5 µL SYBR Premix Ex Taq (Takara, Dalian, China). Parallel reactions to amplify *actin* were used to normalize the amount of template. Relative expression was calculated by $2^{-\Delta\Delta Ct}$. The expression levels in the control plants without treatment (0 h) were normalized to 1. The *MtActin* gene was used as the internal control. The qRT-PCR primers listed in Table S6 were acquired by the PrimerQuest tool (<http://www.idtdna.com/Primerquest/Home/Index>).

5. Conclusions

A total of 50 *MtCML* genes were identified in *M. truncatula*, which were divided into eight groups. Analysis of intron–exon structure revealed that *CML* gene family is evolutionarily conserved. The functional divergence between subgroups was mainly attributed to changes in the EF-hand domains, which may confers functional differentiation of *MtCMLs*. Synteny analysis showed that WGD and tandem duplication probably participated in driving *MtCML* genes evolution. Expression profile analysis showed that most of *MtCML* genes are expressed in leaves, flowers and roots and had a tissue and organ expression preference. Many *cis*-acting element responsive to plant hormones, such as ABA, MeJA and auxin were enriched in the promoter of *MtCMLs*, while salicylic acid (TCA-motif), gibberellin (GARE-motif), circadian, drought (MBS), and cold (LTR) response elements were found in the promoter of some *MtCMLs*. Many *MtCMLs* were regulated by drought and salt stresses. qRT-PCR analysis showed that *MtCML16* and *MtCML33* were upregulated, *MtCML2*, *MtCML6*, *MtCML19* and *MtCML47* were downregulated in response to cold treatment. Our results provide new information for further understanding of the structure, evolution and function of *MtCML* gene family.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1422-0067/21/19/7142/s1>. Table S1. The CML protein sequence in *Medicago truncatula*. Table S2. The expression pattern of *MtCML* genes in different developmental tissues of *Medicago truncatula*. Table S3. The expression level of the *MtCML* genes under salt and drought stresses from the microarray data. Table S4. The relative expression of *MtCML* genes in cold treatment. Table S5. The promoter information of *MtCML* genes. Table S6. Sequences of primers used in qRT-PCR. Table S7. The percentages of identity between pairs of paralogous *MtCML* proteins.

Author Contributions: Z.G. and Q.S. designed this study; Q.S. and S.Y. performed the bioinformatics analysis; Z.G. monitored the experimental work; Q.S. and S.Y. carried out the expression analysis; Q.S. and Z.G. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Natural Science Foundation (Grant No. 32030074).

Conflicts of Interest: The authors declare no conflict of interest.

References

- Kudla, J.; Batistic, O.; Hashimoto, K. Calcium signals: The lead currency of plant information processing. *Plant Cell* **2010**, *22*, 541–563. [[CrossRef](#)]
- Leba, L.J.; Perochon, A.; Cheval, C.; Ranty, B.; Galaud, J.P.; Aldon, D. CML9, a multifunctional *Arabidopsis thaliana* calmodulin-like protein involved in stress responses and plant growth? *Plant Signal Behav.* **2012**, *7*, 1121–1124. [[CrossRef](#)]
- Batistic, O.; Kudla, J. Analysis of calcium signaling pathways in plants. *Biochim. Biophys. Acta* **2012**, *1820*, 1283–1293. [[CrossRef](#)]
- Shi, S.; Li, S.; Asim, M.; Mao, J.; Xu, D.; Ullah, Z.; Liu, G.; Wang, Q.; Liu, H. The *Arabidopsis* calcium-dependent protein kinases (CDPKs) and their roles in plant growth regulation and abiotic stress responses. *Int. J. Mol. Sci.* **2018**, *19*, 1900. [[CrossRef](#)]
- Viridi, A.S.; Singh, S.; Singh, P. Abiotic stress responses in plants: Roles of calmodulin-regulated proteins. *Front. Plant Sci.* **2015**, *6*, 809. [[CrossRef](#)]
- Zhu, J. Abiotic stress signaling and responses in plants. *Cell* **2016**, *167*, 313–324. [[CrossRef](#)]
- Day, I.S.; Reddy, V.S.; Shad, A.G.; Reddy, A.S. Analysis of EF-hand-containing proteins in *Arabidopsis*. *Genome Biol.* **2002**, *3*, H0056. [[CrossRef](#)] [[PubMed](#)]
- McCormack, E.; Tsai, Y.; Braam, J. Handling calcium signaling: *Arabidopsis* CaMs and CMLs. *Trends Plant Sci.* **2005**, *10*, 383–389. [[CrossRef](#)] [[PubMed](#)]
- McCormack, E.; Braam, J. Calmodulins and related potential calcium sensors of *Arabidopsis*. *New Phytol.* **2003**, *159*, 585–598. [[CrossRef](#)]
- 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. [[CrossRef](#)]
- Dubrovina, A.S.; Aleynova, O.A.; Ogneva, Z.V.; Suprun, A.R.; Ananov, A.A.; Kiselev, K.V. The effect of abiotic stress conditions on expression of calmodulin (*CAM*) and calmodulin-like (*CML*) genes in wild-growing grapevine *Vitis amurensis*. *Plants* **2019**, *8*, 602. [[CrossRef](#)] [[PubMed](#)]

12. 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*, 842. [[CrossRef](#)] [[PubMed](#)]
13. Munir, S.; Khan, M.R.; Song, J.; Munir, S.; Zhang, Y.; Ye, Z.; Wang, T. Genome-wide identification, characterization and expression analysis of calmodulin-like (CML) proteins in tomato (*Solanum lycopersicum*). *Plant Physiol. Biochem.* **2016**, *102*, 167–179. [[CrossRef](#)] [[PubMed](#)]
14. Perochon, A.; Aldon, D.; Galaud, J.P.; Ranty, B. Calmodulin and calmodulin-like proteins in plant calcium signaling. *Biochimie* **2011**, *93*, 2048–2053. [[CrossRef](#)] [[PubMed](#)]
15. Dobney, S.; Chiasson, D.; Lam, P.; Smith, S.P.; Snedden, W.A. The calmodulin-related calcium sensor CML42 plays a role in trichome branching. *J. Biol. Chem.* **2009**, *284*, 31647–31657. [[CrossRef](#)] [[PubMed](#)]
16. Ogunrinde, A.; Munro, K.; Davidson, A.; Ubaid, M.; Snedden, W.A. *Arabidopsis* calmodulin-like proteins, CML15 and CML16 possess biochemical properties distinct from calmodulin and show non-overlapping tissue expression patterns. *Front. Plant Sci.* **2017**, *8*, 2175. [[CrossRef](#)]
17. Zeng, H.; Xu, L.; Singh, A.; Wang, H.; Du, L.; Poovaiah, B.W. Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses. *Front. Plant Sci.* **2015**, *6*, 600. [[CrossRef](#)]
18. Dodd, A.N.; Kudla, J.; Sanders, D. The language of calcium signaling. *Annu. Rev. Plant Biol.* **2010**, *61*, 593–620. [[CrossRef](#)]
19. Das, R.; Pandey, G.K. Expressional analysis and role of calcium regulated kinases in abiotic stress signaling. *Curr. Genom.* **2010**, *11*, 2–13. [[CrossRef](#)]
20. Tsai, Y.C.; Delk, N.A.; Chowdhury, N.I.; Braam, J. *Arabidopsis* potential calcium sensors regulate nitric oxide levels and the transition to flowering. *Plant Signal Behav.* **2007**, *2*, 446–454. [[CrossRef](#)]
21. Yang, X.; Wang, S.S.; Wang, M.; Qiao, Z.; Bao, C.C.; Zhang, W. *Arabidopsis thaliana* calmodulin-like protein CML24 regulates pollen tube growth by modulating the actin cytoskeleton and controlling the cytosolic Ca²⁺ concentration. *Plant Mol. Biol.* **2014**, *86*, 225–236. [[PubMed](#)]
22. Wang, Y.; Wang, B.; Gilroy, S.; Wassim Chehab, E.; Braam, J. CML24 is involved in root mechanoresponses and cortical microtubule orientation in *Arabidopsis*. *J. Plant Growth Regul.* **2011**, *30*, 467–479.
23. Delk, N.A.; Johnson, K.A.; Chowdhury, N.I.; Braam, J. CML24, regulated in expression by diverse stimuli, encodes a potential Ca²⁺ sensor that functions in responses to abscisic acid, daylength, and ion stress. *Plant Physiol.* **2005**, *139*, 240–253. [[PubMed](#)]
24. Marti, R.M.; Hubbard, K.E.; Gardner, M.J.; Jung, H.J.; Aubry, S.; Hotta, C.T.; Mohd-Noh, N.I.; Robertson, F.C.; Hearn, T.J.; Tsai, Y.C.; et al. Circadian oscillations of cytosolic free calcium regulate the *Arabidopsis* circadian clock. *Nat. Plants* **2018**, *4*, 690–698.
25. Wang, S.S.; Diao, W.Z.; Yang, X.; Qiao, Z.; Wang, M.; Acharya, B.R.; Zhang, W. *Arabidopsis thaliana* CML25 mediates the Ca²⁺ regulation of K⁺ transmembrane trafficking during pollen germination and tube elongation. *Plant Cell Environ.* **2015**, *38*, 2372–2386.
26. Zha, G.; Wang, B.; Liu, J.; Yan, J.; Zhu, L.; Yang, X. Mechanical touch responses of *Arabidopsis TCH1-3* mutant roots on inclined hard-agar surface. *Int. Agrophys.* **2016**, *30*, 105–111.
27. Bender, K.W.; Dobney, S.; Ogunrinde, A.; Chiasson, D.; Mullen, R.T.; Teresinski, H.J.; Singh, P.; Munro, K.; Smith, S.P.; Snedden, W.A. The calmodulin-like protein CML43 functions as a salicylic-acid-inducible root-specific Ca²⁺ sensor in *Arabidopsis*. *Biochem. J.* **2014**, *457*, 127–136.
28. Midhat, U.; Ting, M.; Teresinski, H.J.; Snedden, W.A. The calmodulin-like protein, CML39, is involved in regulating seed development, germination, and fruit development in *Arabidopsis*. *Plant Mol. Biol.* **2018**, *96*, 375–392.
29. Campos, W.F.; Dressano, K.; Ceciliato, P.; Guerrero-Abad, J.C.; Silva, A.L.; Fiori, C.S.; Morato, D.C.A.; Bergonci, T.; Claus, L.; Silva-Filho, M.C.; et al. *Arabidopsis thaliana* rapid alkalization factor 1-mediated root growth inhibition is dependent on calmodulin-like protein 38. *J. Biol. Chem.* **2018**, *293*, 2159–2171.
30. Lu, Y.; Truman, W.; Liu, X.; Bethke, G.; Zhou, M.; Myers, C.L.; Katagiri, F.; Glazebrook, J. Different modes of negative regulation of plant immunity by calmodulin-related genes. *Plant Physiol.* **2018**, *176*, 3046–3061.
31. Xu, B.; Cheval, C.; Laohavisit, A.; Hocking, B.; Chiasson, D.; Olsson, T.; Shirasu, K.; Faulkner, C.; Gilliam, M. A calmodulin-like protein regulates plasmodesmal closure during bacterial immune responses. *New Phytol.* **2017**, *215*, 77–84. [[CrossRef](#)] [[PubMed](#)]

32. Zhu, X.; Robe, E.; Jomat, L.; Aldon, D.; Mazars, C.; Galaud, J.P. CML8, an Arabidopsis calmodulin-like protein, plays a role in *Pseudomonas syringae* plant immunity. *Plant Cell Physiol.* **2017**, *58*, 307–319. [[PubMed](#)]
33. Vadassery, J.; Reichelt, M.; Hause, B.; Gershenzon, J.; Boland, W.; Mithofer, A. CML42-mediated calcium signaling coordinates responses to *Spodoptera* herbivory and abiotic stresses in Arabidopsis. *Plant Physiol.* **2012**, *159*, 1159–1175. [[CrossRef](#)]
34. Scholz, S.S.; Reichelt, M.; Vadassery, J.; Mithofer, A. Calmodulin-like protein CML37 is a positive regulator of ABA during drought stress in Arabidopsis. *Plant Signal Behav.* **2015**, *10*, e1011951. [[CrossRef](#)] [[PubMed](#)]
35. Vanderbeld, B.; Snedden, W.A. Developmental and stimulus-induced expression patterns of Arabidopsis calmodulin-like genes *CML37*, *CML38* and *CML39*. *Plant Mol. Biol.* **2007**, *64*, 683–697. [[CrossRef](#)]
36. Wu, X.; Qiao, Z.; Liu, H.; Acharya, B.R.; 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. [[CrossRef](#)] [[PubMed](#)]
37. Jung, H.; Chung, P.J.; Park, S.H.; Redillas, M.; Kim, Y.S.; Suh, J.W.; Kim, J.K. Overexpression of *OsERF48* causes regulation of *OsCML16*, a calmodulin-like protein gene that enhances root growth and drought tolerance. *Plant Biotechnol. J.* **2017**, *15*, 1295–1308. [[CrossRef](#)]
38. Chen, C.; Sun, X.; Duanmu, H.; Zhu, D.; Yu, Y.; Cao, L.; Liu, A.; Jia, B.; Xiao, J.; Zhu, Y. *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* **2015**, *10*, e0141888. [[CrossRef](#)]
39. Young, N.D.; Debelle, F.; Oldroyd, G.E.; Geurts, R.; Cannon, S.B.; Udvardi, M.K.; Benedito, V.A.; Mayer, K.F.; Gouzy, J.; Schoof, H.; et al. The *Medicago* genome provides insight into the evolution of rhizobial symbioses. *Nature* **2011**, *480*, 520–524. [[CrossRef](#)]
40. Wang, T.; Liu, M.; Sun, W.; Zhang, X.; Zhang, W. Calmodulin-like gene *MtCML40* is involved in salt tolerance by regulating MthKTs transporters in *Medicago truncatula*. *Environ. Exp. Bot.* **2019**, *157*, 79–90.
41. Zhang, Z.; Hu, X.; Zhang, Y.; Miao, Z.; Xie, C.; Meng, X.; Deng, J.; Wen, J.; Mysore, K.S.; Frugier, F.; et al. Opposing Control by Transcription Factors MYB61 and MYB3 Increases Freezing Tolerance by Relieving C-Repeat Binding Factor Suppression. *Plant Physiol.* **2016**, *172*, 1306–1323. [[CrossRef](#)] [[PubMed](#)]
42. Li, D.; Su, Z.; Dong, J.; Wang, T. An expression database for roots of the model legume *Medicago truncatula* under salt stress. *BMC Genom.* **2009**, *10*, 517. [[CrossRef](#)] [[PubMed](#)]
43. Mohanta, T.K.; 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. [[CrossRef](#)] [[PubMed](#)]
44. Bergey, D.R.; Kandel, R.; Tyree, B.K.; Dutt, M.; Dhekney, S.A. The role of calmodulin and related proteins in plant cell function: An ever-thickening plot. *Springer Sci. Rev.* **2014**, *2*, 145–159. [[CrossRef](#)]
45. Freeling, M. Bias in plant gene content following different sorts of duplication: Tandem, whole-genome, segmental, or by transposition. *Annu. Rev. Plant. Biol.* **2009**, *60*, 433–453. [[CrossRef](#)] [[PubMed](#)]
46. Cannon, S.B.; Ilut, D.; Farmer, A.D.; Maki, S.L.; May, G.D.; Singer, S.R.; Doyle, J.J. Polyploidy did not predate the evolution of nodulation in all legumes. *PLoS ONE* **2010**, *5*, e11630. [[CrossRef](#)] [[PubMed](#)]
47. Pfeil, B.E.; Schlueter, J.A.; Shoemaker, R.C.; Doyle, J.J. Placing paleopolyploidy in relation to taxon divergence: A phylogenetic analysis in legumes using 39 gene families. *Syst. Biol.* **2005**, *54*, 441–454. [[CrossRef](#)]
48. Wang, Y.; Zhang, W.Z.; Song, L.F.; Zou, J.J.; Su, Z.; Wu, W.H. Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in Arabidopsis. *Plant Physiol.* **2008**, *148*, 1201–1211. [[CrossRef](#)]
49. Magnan, F.; Ranty, B.; Charpentreau, M.; Sotta, B.; Galaud, J.P.; 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*, 575–589.
50. Xu, G.Y.; Rocha, P.S.; Wang, M.L.; Xu, M.L.; Cui, Y.C.; Li, L.Y.; Zhu, Y.X.; Xia, X. A novel rice calmodulin-like gene, *OsMSR2*, enhances drought and salt tolerance and increases ABA sensitivity in Arabidopsis. *Planta* **2011**, *234*, 47–59. [[CrossRef](#)]
51. Munir, S.; Liu, H.; Xing, Y.; Hussain, S.; Ouyang, B.; Zhang, Y.; Li, H.; Ye, Z. Overexpression of calmodulin-like (*ShCML44*) stress-responsive gene from *Solanum habrochaites* enhances tolerance to multiple abiotic stresses. *Sci. Rep.* **2016**, *6*, 31772. [[CrossRef](#)] [[PubMed](#)]
52. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [[CrossRef](#)] [[PubMed](#)]

53. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant* **2020**, *13*, 1194–1202. [[CrossRef](#)] [[PubMed](#)]
54. Lescot, M.; Dehais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van de Peer, Y.; Rouze, P.; Rombauts, S. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327. [[CrossRef](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).