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

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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 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).

| 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    | 8.67 | −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| Medtr3g086655.1 | 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  |
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.

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 | −0.32 |
| 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 | Medtr7g011101.1   | 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 | −0.35 |
| 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 hydrophaticity.
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).
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.
was major expressed in leaves and flowers as well as in stems, but not in roots and pods (Figure 4E),

were found with 1–3 paralogous genes in Medicago truncatula.

Among Medicago truncatula, three genes (MtCML17, MtCML14 and MtCML22) were preferentially expressed in roots, and its transcript could be detected in stems, flowers and pods (Figure 4B). 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),

Figure 3. Chromosomal distribution and synteny analysis of CML genes in the genomes of Medicago truncatula and Arabidopsis thaliana. (A) Paralogous MiCML 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 MiCML gene pairs.

Collinearity diagrams among MiCMLs were further analyzed. The results showed that all MiCML genes underwent gene duplication in M. truncatula genome (Figure 3A, Table S7). Most MiCML genes were found with 1–3 paralogous genes in M. truncatula. Five MiCML genes (MiCML5, MiCML6, MiCML8, MiCML12 and MiCML20) 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 (MiCML7, MiCML27 and MiCML50) had two homologous genes in A. thaliana, while two genes (MiCML12 and MiCML18) had three orthologous genes in A. thaliana. The percentage of identity between pairs of paralogous MiCML 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 MiCML protein sequences and functions could be highly conserved. These results indicated that MiCML 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 MiCMLs

The spatial and temporal expression profiles of MiCMLs were examined based on the microarray data from M. truncatula Gene Expression Atlas (MiGEA, https://mtgea.noble.org/v3/). Twenty-nine MiCMLs have corresponding probe sets in the dataset (Table S2). MiCML13, MiCML27, MiCML36, MiCML38 and MiCML42 exhibited relatively high expression in all tissues (flowers, leaves, petioles, pods, stems, roots and seeds) (Figure 4A), indicating that their functions may be extensive. MiCML7, MiCML14, MiCML17 and MiCML22 were preferentially expressed in roots, and MiCML47 was only expressed in mature seeds, while MiCML20 and MiCML30 were expressed in leaves. Some MiCMLs (MiCML6, MiCML13, MiCML25, MiCML28 and MiCML30) were highly expressed in floral organs (Figure 4A). On the other hand, relative expression of MiCML7, MiCML14, MiCML17, MiCML20, MiCML22 and MiCML47 was examined using qRT-PCR (Figure 4B–G). MiCML7 was mostly expressed in roots, stems and leaves, but not in seeds (Figure 4B); MiCML14 was highly expressed in leaves and its transcript could be detected in roots, stems, flowers and seeds (Figure 4C). MiCML17 was highly expressed in roots, and its transcript could be detected in stems, flowers and pods (Figure 4D). MiCML20 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; circadin—*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]. MtCML 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 rewetering, while MtCML7, MtCML14, MtCML22, MtCML37, MtCML39, MtCML45 and MtCML49 transcripts were significantly reduced after drought treatment followed by increase after rewetering (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*. 

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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/).
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

**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 [31]. 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 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 $2^{-\Delta\DeltaCT}$. 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.

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