Plant Physiology and Biochemistry 139 (2019) 600–612

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Genome-wide identification and expression analysis of calmodulin and calmodulin-like genes in apple (*Malus* × *domestica*)

Chunlong Li[a,1], Dong Meng[b,1], Junhong Zhang[a,c], Lailiang Cheng[a,*]

[a] School of Integrative Plant Science, Cornell University, 134A Plant Science, Ithaca, NY, 14853, USA
[b] Beijing Advanced Innovation Center for Tree Breeding By Molecular Design, Beijing Forestry University, Beijing, China
[c] State Key Laboratory of Subtropical Silviculture, Zhejiang Agriculture and Forestry University, Lin'an, Hangzhou, Zhejiang, PR China

ARTICLE INFO

Keywords:
Apple (*Malus* × *domestica*)
Calcium
Calmodulin
Calmodulin-like

ABSTRACT

Changes in intracellular calcium (Ca^{2+}) levels in response to developmental processes or external stimuli serve as signals in eukaryotic cells. These Ca^{2+} signals are likely perceived through sensor proteins that bind Ca^{2+} by EF-hand (a helix-loop-helix structure) motif. Calmodulins (CaMs), a group of well-characterized Ca^{2+} sensors, and calmodulin-like (CMLs) are implicated in a large number of diverse cellular processes, including plant development and stress responses. In this study, apple (*Malus* × *domestica*) genes encoding CaM and CML proteins that only possess EF-hand motifs with no other functional domains were analyzed. A total of 4 *MdCaM* and 58 MdCML genes were identified, which are spread among 16 out of the 17 apple chromosomes. Bioinformatics analyses, including protein characteristics, conserved domain, evolutionary relationships and chromosomal locations, demonstrated the conservation and divergence of MdCaMs/CMLs. In addition, expression analysis showed that *MdCaMs/CMLs* are expressed in more than one tissue, including shoot tips, roots, mature leaves, flowers and fruit. Furthermore, the expression of some *MdCaM/CML* members responded to plant hormones (abscisic acid, jasmonic acid) and salt stress, suggesting a potential role of these genes in responses to biotic and abiotic stress. Overexpression of stress-induced *MdCML3* gene significantly improved the tolerance of apple calli to salinity and ABA. The identification and characterization of *MdCaMs/CMLs* in apple lays a foundation for future functional studies of these genes.

1. Introduction

Calcium (Ca^{2+}), as a major second messenger, is involved in responses of all eukaryotic cells to internal signal transduction and external stimuli (Berridge et al., 2000; Dodd et al., 2010; Hetherington and Brownlee, 2004). The associated changes in intracellular Ca^{2+} concentrations, presented as calcium signatures, are typically amplified by protein sensors that preferably bind Ca^{2+} (Batistic and Kudla, 2012; DeFalco et al., 2009; Hashimoto and Kudla, 2011). These Ca^{2+}-binding proteins sensors are divided into four distinct groups, namely the calmodulins (CaMs), the CaM-like proteins (CMLs), the Ca^{2+}-dependent protein kinases (CDPKs) and the calcineurin B-like proteins (CBLs) (Cheng et al., 2002; Luan, 2009; McCormack et al., 2005; Weinl and Kudla, 2009). For the majority of Ca^{2+} sensors, the EF-hand (a helix loop-helix structure) is the most frequent motif identified as the Ca^{2+}-binding site. Ca^{2+} binding leads to a change in protein conformation, amplifying the signal by modulating their activity or their ability to interact with downstream proteins (Gifford et al., 2007; Grabarek, 2006; Lewit-Bentley and Rety, 2000).

Among the Ca^{2+}-binding proteins, Calmodulin-like proteins (CMLs) belong to a plant-specific family of Ca^{2+} sensors. They are featured by Ca^{2+}-binding EF hands and share at least 16% amino acid identity with calmodulin (McCormack et al., 2005; Perochon et al., 2011; Zhu et al., 2015). CaM is a highly conserved Ca^{2+}-binding protein in eukaryotes, and typically contains four EF-hand motifs. However, there are one to six EF-hand motifs in CMLs in comparison with CaM. Additionally, plants encode more CMLs than CaMs in general genome-wide identification of *CaM* and *CML* gene family members (Mohanta et al., 2017). A total of 50 and 32 members of the CML family have been reported in *Arabidopsis* and rice, respectively, but the corresponding numbers of CaM members are only seven and five (Boonburapong and Buaboocha, 2007; McCormack et al., 2005). Besides EF-hands, CaMs and CMLs do not have any other known functional domain, which is different from the members of CDPKs and CBLs.

* Corresponding author.
E-mail address: LC89@cornell.edu (L. Cheng).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.plaphy.2019.04.014
Received 24 November 2018; Received in revised form 11 April 2019; Accepted 11 April 2019
Available online 17 April 2019

As essential Ca^{2+} sensors, CaMs and CMLs are involved in plant developmental processes and responses to biotic and abiotic stresses. For example, AtCML24 and AtCML25 play vital roles in pollen germination and pollen tube growth (Wang et al., 2015; Yang et al., 2014). *cml24* mutant has significantly reduced pollen tube growth (Yang et al., 2014), and CML25 is involved in the Ca^{2+}-mediated regulation of K^+ influx during pollen germination and tube elongation (Wang et al., 2015). A number of previous reports indicate that the expression of CaM and CML genes strongly affects the plant immune system (Ranty et al., 2016). In tobacco, type III CaM isoforms are probably involved in basal defense against necrotrophic pathogens, which is independent of jasmonic acid (JA) and ethylene signaling (Takabatake et al., 2007). *AtCML9* expression was induced in plants exposed to bacterial pathogen and to application of stress-associated phytohormones such as abscisic acid (ABA) and salicylic acid (SA), and AtCML9 was involved in plant defence by modulating responses to bacterial strains of *Pseudomonas syringae* (Leba et al., 2012; Magnan et al., 2008). The infection of *P. syringae* also strongly and transiently induced the expression of *AtCML8*, which serves as a positive regulator for SA-dependent processes of plant immunity (Zhu et al., 2017). In cotton, *GhCML11* participated in the defense response against *Verticillium dahliae* infection under the regulation of transcription factor *GhMYB108* (Cheng et al., 2016).

CaMs and CMLs are also involved in plant abiotic responses, such as salinity, drought, and heat stresses (Zeng et al., 2015). Overexpression of a salt-induced CaM gene *GmCaM4* in *Arabidopsis* confers salinity tolerance through the up-regulation of a MYB transcription factor, MYB2 (Yoo et al., 2005). Ectopic expression of *GsCML27* in *Arabidopsis* decreased salinity and osmotic tolerance during seed germination and early growth stages by modifying both the cellular ionic (Na^+, K^+) concentration and regulating the transcript levels of osmotic stress responsive genes (Chen et al., 2015). A novel calmodulin-like protein gene isolated from rice, *OsMSR2*, was reported to be involved in ABA-mediated salinity and drought tolerance (Xu et al., 2011). There is also a crosstalk between CaM/CML and another important second messenger, reactive oxygen species (ROS), in plant response to abiotic stress (Ozgur et al., 2014; Suzuki et al., 2012). The ABA-sensitive stomatal movement and drought-resistance phenotypes were reported in *Arabidopsis* mutant *cml20*, which is accompanied with the down-regulated *APX2* transcription and higher ROS accumulation in guard cells (Wu et al., 2017). The latest research on grapevine (*Vitis vinifera*) CaMs/CMLs gene family also demonstrated that many members' expression was up or down-regulated under various abiotic stresses (drought, heat, gibberellic acid, abscisic acid or UV-C treatment), which suggests multiple function of *VviCaMs/CMLs* in grapevine stress resistance (Vandelle et al., 2018).

In consideration of their important roles, genes encoding CaMs/CMLs have been analyzed at the whole genome scale in various model plants and crops (Boonburapong and Buaboocha, 2007; Liao et al., 2017; Mohanta et al., 2017; Munir et al., 2016; Nie et al., 2017; Vandelle et al., 2018; Zhang et al., 2016; Zhao et al., 2013). However, CaMs/CMLs genes have not been identified and characterized systematically in apple, an economically important fruit crop cultivated worldwide (Cornille et al., 2014). Moreover, understanding Ca^{2+} as a second messenger is particularly relevant because many apple cultivars are susceptible to bitter pit, a physiological disorder related to Ca^{2+} deficiency, and both pre- and post-harvest applications of Ca^{2+} can reduce the incidence of bitter pit (de Freitas and Mitcham, 2012; Ferguson and Watkins, 1989). Characterization and expression analysis of the CPK gene family in apple cultivars with contrasting susceptibility to fire blight and comparisons with other Rosaceae species demonstrated that MdCPKs play important roles in responses to *Erwinia amylovora* and *Alternaria alternata* infection (Kanchiswamy et al., 2013; Wei et al., 2016). However, compared with the extensive and in-depth work on Ca^{2+} sensors in other plants, understanding of the *CaMs/CMLs* gene family in apple is very limited.

In this study, we identified 4 *CaM* genes and 58 CML genes in the apple (*Malus* × *domestica*) genome and determined their expression profiles in different tissues, during fruit development, and in response to hormone treatments. qRT-PCR was also used to detect the expression levels of highly expressed members in response to salinity stress and ABA treatment. One such gene, *MdCML3*, when overexpressed in apple calli, significantly improved their tolerance to salinity stress and ABA treatment, demonstrating that identification and expression analysis of *MdCaMs/CMLs* in apple lays a foundation for functional characterization of these genes in stress tolerance and developmental processes.

2. Materials and methods

2.1. Database searches and sequence annotation for identification of the CaM and CML family in apple

To identify CaM and CML proteins in apple, the sequences of *Arabidopsis* CaM and CML proteins were downloaded from the *Arabidopsis* genome (TAIR, http://www.Arabidopsis.org/). These sequences were used as queries for BLASTP (with an E cut off value 1e-5) search of the apple genome sequence database on the website GDR (Genome Database for *Rosaceae*, https://www.rosaceae.org/). In addition, we used 'Calmodulin', 'EF-hand', and 'Calmodulin-like protein' as keywords to perform homolog searches in the apple genome database. All the *MdCaM/CML* genes were named consecutively based on GeneID number in the Genome Database (GDR, https://www.rosaceae.org/) and chromosomal position. Nucleotide and amino acid sequences as well as genes' related information were obtained. Redundant sequences or sequences lacking the EF-hand domain were removed after a similarity comparison, and subsequent analyses were performed using Hidden Markov Model of Simple Modular Architecture Research Tool SMART (http://smart.embl-heidelberg.de/), InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/), Pfam (http://pfam.sanger.ac.uk/) and NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd) programs to verify the reliability of MdCaM and MdCML candidate sequences.

2.2. Phylogenetic tree construction and chromosomal location analysis

Phylogenetic analysis was conducted using MEGA version 6.0 (http://www.megasoftware.net/) with the neighbor-joining method, with bootstrap values calculated using 1000 iterations. Sixty two genes from apple and 56 genes from *Arabidopsis* were used. The chromosome location of the *MdCaM* and *MdCML* genes was performed using MapChart program based on gene position and markers in the apple genome supplied by the GDR website (https://www.rosaceae.org/).

2.3. Amino acid identity and motif analyses of proteins

Protein physiochemical data, including the theoretical isoelectric point (pI), molecular weight, and sequence length were obtained on the ExpasyProtParam server (http://web.expasy.org/protparam/). Structural motif annotation was performed using the MEME program (http://meme-suite.org/) (Bailey and Elkan, 1994). Protein subcellular localization was predicted via the Wolf PSORT II program (https://www.genscript.com/wolf-psort.html?src=leftbar).

2.4. Plant materials and growth conditions

Growing shoot tips, mature leaves, flowers, stamens, and fruit at five developmental stages from cell division (S1) to ripening (S5) (S1: 18 DAB, active cell division; S2: 37 DAB, end of cell division; S3: 67 DAB, early rapid cell expansion; S4: 90 DAB, late rapid cell expansion; S5: 132 DAB, ripening) were obtained from 'Greensleeves'/M.26 apple trees in 3–5 replicates, and white roots from 'G.890' rootstock were taken during active root growth in 5 replicates for mRNA isolation and

RNA-seq library construction. The vegetative tissues (roots, shoot tips and leaves) and reproductive organs (flower, stamens, and fruits) were sampled from 2-year old trees and 5-year old trees grown in containers at Cornell Orchards, respectively, as described before (Li et al., 2018; Meng et al., 2018a; Wu et al., 2015). For the ABA and JA feeding experiment for RNAseq analysis, two-year old 'Greensleeves' trees on M.26 rootstock were grown in 7.6 L containers at Cornell Orchards as previously described (Cheng et al., 2005). Leaves were fed with exogenous ABA or JA via transpiration stream following Meng et al. (2018b) for sugar feeding with modifications (Meng et al., 2018b). The youngest fully expanded leaves were cut at the petiole base from the shoots with a razor blade, and immediately re-cut under water. These leaves were randomly assigned to ABA (1 μM) treatment, JA (10 μM) treatment or water control, with five leaves per replicate. The petiole of each leaf was immersed into ABA/JA solution or water in a 2-ml Eppendorf vial (wrapped in aluminum foil) inserted into a piece of Styrofoam in an angle that allows the leaf blade to be perpendicular to the lights. Each treatment was replicated five times, and they were randomly arranged in a fume hood (2 m × 1.2 m × 1.5 m) under fluorescent lights at ~70 μmol photons m^{-2} s^{-1} at 23 °C. Leaf samples were collected at 3 h after ABA/JA feeding was initiated, frozen in liquid nitrogen and stored at −80 °C for use.

Tissue-cultured plantlets of 'Greensleeves' apple (*Malus domestica* Borkh) were used in the qRT-PCR analysis. They were grown on MS medium at 23 °C under fluorescent lights at ~50 μmol photons m^{-2} s^{-1} in a 16-hr photoperiod. After one month in subculture, uniform plants were selected for salinity or ABA treatment. The treated plants were drenched with 200 mM NaCl or 50 μM ABA, with water as control. Each treatment was replicated 5 times with 3 plants per replicate in a completely randomized design. Samples were taken at 0, 2, 6, 12, and 24 h after the treatments, and frozen in liquid nitrogen for later use.

2.5. Library construction and RNA-Seq analyses

RNA-seq library construction, sequencing and data analysis for apple leaves, flowers, and fruits were described previously (Duan et al., 2017; Meng et al., 2018a; Wu et al., 2015), and the same protocols were used for constructing the RNAseq libraries, sequencing and data analysis for apple roots, shoot tips, stamens, and leaves fed with ABA, JA or water. Heatmaps showing the expression profiles of *MdCaM* and *MdCML* genes were constructed using the MeV software (http://mev.tm4.org/) based on the RPKM (reads per kilobase of exon model per million mapped reads) value of each gene obtained in the RNA-seq analyses to compare their expression levels between different tissues, at different developmental stages for apple fruit, and responses to ABA and JA treatments.

2.6. RNA isolation and qRT-PCR

Frozen apple tissues were ground in liquid nitrogen to a fine powder, and RNA was extracted using the CTAB method as described (Meng et al., 2014). After treatment with RQ1 DNase (Promega, Madison, WI, USA), RNA concentration was measured by using a NanoDrop spectrophotometer, and RNA integrity was confirmed by agarose gel electrophoresis. One microgram of total RNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed in 3 replicates on an Icycler iQ5 (BioRad) using the SYBR Green Supermix kit (Bio-Rad) according to the instruction manual. All primer sequences are given in Supplemental Table 1. Data were analyzed by the iQ5 2.0 software (Bio-Rad) using the $2^{-\Delta\Delta CT}$ method.

2.7. Apple callus transformation and stress treatments

'Orin' apple calli were used for genetic transformation. They were cultured on MS medium with 30 g/L sucrose, 1.5 mg/L 6-BA and 0.5 mg/L indole-3-acetic acid at 25 °C in the dark. Transformation was performed as described previously with slight modifications (An et al., 2012). Full-length *MdCML3* (MDP0000215758) cDNA was amplified using primers MdCML3-GW-F/R (Supplemental Table 1), and cloned into the Gateway pGWB417 vector through Gateway BP and LR reactions (Invitrogen; BP Clonase; LR Clonase). Then the construct and empty vector were transformed into *Agrobacterium* GV3101, respectively, for apple callus transformation. Three-week old 'Orin' apple calli were collected, and then suspended in the liquid medium containing GV3101 for 15 min under shaking at 140 rpm. Subsequently, the calli were co-cultured on solid MS medium (4.43 g/L MS, 1.0 mg/L 6-BA, 1.0 mg/L 2, 4-D, 30 g/L sucrose, 7 g/L agar, pH 5.8) at 25 °C in the dark. After 2–3 days, the calli were washed three times with sterile water, and then cultured on the solid MS medium containing 250 mg/L carbenicillin and 30 mg/L kanamycin for selection. The *MdCML3* overexpression transgenic calli were identified through qRT-PCR. For stress tolerance assays, the transgenic apple calli were cultured on MS medium for 10 days, and then were transferred to MS medium with 100 mM NaCl or 10 μMABA for two weeks. Each treatment was replicated three times.

3. Results

3.1. Identification of CaM and CML family genes in apple

To identify *CaM* and *CML* genes in the apple genome, *Arabidopsis CaMs/CMLs* genes were downloaded from TAIR database (http://www.arabidopsis.org/) and used as query sequences. All of the redundant genes were removed after a similarity sequence comparison. Finally, a total of 4 *MdCaM* and 58 MdCML genes were identified and annotated (Table 1). Based on GeneID number in GDR (https://www.rosaceae.org/) and chromosomal position, all the MdCaM/CML genes were named consecutively from *MdCaM1* to 4 and *MdCML1* to 58, respectively (Table 1). The protein sequences corresponding to the identified *MdCaM* and *MdCML* genes were analyzed through Pfam (http://pfam.sanger.ac.uk/) and InterproScan platform (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) to confirm the presence of the EF-hand domain and protein characteristics, which include the number of amino acids (AA), theoretical isoelectric point, and molecular weight. As shown in Table 1, all of the MdCaMs had four EF-hands domain, and MdCMLs contained two to four EF-hands. The sizes of these proteins ranged from 123 (MdCML52) to 709 (MdCML34) AA residues, with molecular weight from 13.7 (MdCML28) to 80.7 (MdCML34) KDa. To predict subcellular locations of the MdCaM/CML proteins, we analyzed their sequences using the Wolf PSORT II program. Most of the MdCaMs/CMLs are predicted to be nuclear and cytosolic proteins, but some are plastid or membrane proteins, such as chloroplast proteins, mitochondrial proteins, plasma membrane proteins and vacuolar membrane proteins (Table 1). The various locations suggest diverse functions of these proteins in calcium signaling transduction.

3.2. Sequence alignment and phylogenetic analysis of MdCaM and MdCML genes

To gain insights into the potential functions of MdCaMs and MdCMLs, we constructed an unrooted phylogenetic tree by using MEGA6.0 following the neighbor-joining method, with selected *Arabidopsis* CaM/CML proteins added as reference proteins. MdCaM/CML proteins were classified into nine subgroups according to their similarities and relationships with *Arabidopsis* members (McCormack and Braam, 2003), and each group contained a diverse number of MdCaM/CML proteins (Fig. 1). Group 7 had the most members (17), followed by group 8 (13 members) and 6 (12 members). Group 2 was the smallest and contained only one member, MdCML41, in comparison with 5 CMLs in *Arabidopsis*. We also performed a phylogenetic analysis of MdCaMs/CMLs with those in tomato ((Boonburapong and

Table 1
Information of *MdCaM* and *MdCML* genes.

Gene name	Gene ID[-1]	Genomic location[-2]	pI[-3]	Mw[-4]	AA[-5]	CDS[-6]	EF Hands[-7]	Predicted location(s) [-8]
MdCaM1	MDP0000234624	chr3:29605590..29606560	4.02	16.86829	148	447	4	cyto: 8.5, cyto_nucl: 7.5, nucl: 3.5
MdCaM2	MDP0000183898	chr7:11224230..11225629	4.11	16.84767	149	450	4	nucl: 4, mito: 4, extr: 3, cyto: 2
MdCaM3	MDP0000203128	chr11:31664205..31665158	4.03	16.80945	148	447	4	cyto_nucl: 7, cyto: 6.5, nucl: 4.5, chlo: 3
MdCaM4	MDP0000277474	chr14:28724909..28726352	4.16	16.96582	149	450	4	mito: 6, nucl: 3, chlo: 2, cyto: 2
MdCML1	MDP0000140330	chr2:2711299..2713599	5.77	43.33716	384	1155	4	chlo: 7, extr: 2, vacu: 2, plas: 1, E.R.: 1
MdCML2	MDP0000859609	chr1:26160754..26161242	4.77	18.15642	162	489	4	cyto: 5, nucl: 4.5, mito: 3, nucl_plas: 3
MdCML3	MDP0000215758	chr2:17162856..17163428	5.25	21.26632	190	573	4	nucl: 6, chlo: 3, cyto: 3, mito: 2
MdCML4	MDP0000306089	chr3:3852877..3854078	4.64	26.17483	227	684	4	chlo: 6, cyto: 2, plas: 2, E.R.: 2, mito: 1
MdCML5	MDP0000294713	chr3:15147838..15149897	8.42	52.50028	477	1434	4	nucl: 7, chlo: 3, cyto: 2, plas: 1
MdCML6	MDP0000168498	chr4:440129..442330	8.85	43.79931	385	1158	4	chlo: 14
MdCML7	MDP0000139052	chr4:17852556..17853185	4.66	23.20477	209	630	3	mito: 7.5, chlo_mito: 6, chlo: 3.5, nucl: 3
MdCML8	MDP0000256115	chr4:19342702..19344373	10.45	53.84945	496	1491	2	nucl: 4, E.R.: 3.5, cyto: 2.5, cyto_pero: 2.5, E.R._plas: 2.5
MdCML9	MDP0000120294	chr4:19941933..19944437	6.21	35.00389	335	1008	2	plas: 4.5, nucl: 3, E.R._plas: 3, cyto: 2.5, cyto_pero: 2
MdCML10	MDP0000221350	chr4:21305762..21309403	4.78	32.69916	285	858	4	cyto: 8.5, cyto_E.R.: 5.5, E.R.: 1.5, chlo: 1, mito: 1, plas: 1
MdCML11	MDP0000859814	chr5:25361209..25361880	4.80	24.15500	223	672	4	nucl: 7, chlo: 3, mito: 3
MdCML12	MDP0000606583	chr5:28117985..28118539	5.99	21.34654	184	555	4	chlo: 5, cyto: 4, nucl: 2, mito: 2
MdCML13	MDP0000864163	chr6:3004796..3005287	4.54	17.94899	163	492	4	nucl_plas: 4.5, mito: 4, plas: 4, nucl: 3, cyto: 2
MdCML14	MDP0000545337	chr6:17520591..17521013	4.45	15.6117	140	423	4	nucl: 5, cyto: 5, chlo: 3
MdCML15	MDP0000219283	chr6:21121465..21122100	4.59	23.61393	211	636	2	chlo: 7, nucl: 2, cyto: 2, extr: 1, vacu: 1
MdCML16	MDP0000311043	chr6:22523019..22523501	4.18	16.46163	150	453	4	mito: 4, chlo: 3, cyto: 3, nucl: 2, plas: 2
MdCML17	MDP0000780674	chr6:23785028..23785555	4.45	18.92125	175	528	4	mito: 8, cyto: 3, chlo: 2
MdCML18	MDP0000734433	chr6:27959821..27960363	7.78	16.18846	149	450	2	chlo: 6, nucl: 5, cyto: 2
MdCML19	MDP0000166886	chr7:2232573..2233109	4.38	17.15288	160	483	3	cyto: 8, chlo: 4, mito: 2
MdCML20	MDP0000250594	chr7:12589421..12590030	5.44	20.82411	186	561	4	chlo: 9, mito: 3, nucl: 1
MdCML21	MDP0000146799	chr7:16714199..16715044	4.75	30.8908	281	846	4	chlo: 10.5, chlo_mito: 7, mito: 2.5
MdCML22	MDP0000189381	chr7:21723408..21723967	4.34	19.16088	166	501	2	chlo: 12, mito: 1
MdCML23	MDP0000164511	chr8:3786426..3787001	4.74	21.61466	191	576	3	nucl: 7, mito: 4, chlo: 2
MdCML24	MDP0000586415	chr8:15564618..15565082	4.73	16.90111	154	465	4	nucl: 5, chlo: 3, extr: 3, mito: 2
MdCML25	MDP0000140394	chr8:20364418..20366173	5.65	26.5481	236	711	2	nucl: 9.5, cyto_nucl: 5.5, extr: 3
MdCML26	MDP0000187969	chr8:27827266..27827823	5.5	21.01896	185	558	4	nucl: 12, chlo: 1
MdCML27	MDP0000534175	chr9:901049..901471	4.5	15.52369	140	423	3	chlo: 11, nucl: 3
MdCML28	MDP0000243482	chr9:932608..932988	4.32	13.7648	126	381	3	chlo: 11, nucl: 3
MdCML29	MDP0000432499	chr9:3256551..3257150	4.84	22.1642	199	600	2	chlo: 10, extr: 3
MdCML30	MDP0000190637	chr9:3357800..3358267	4.9	17.49472	155	468	4	cyto: 7, pero: 2, nucl_plas: 2, nucl: 1.5, plas: 1.5, mito: 1
MdCML31	MDP0000307702	chr9:15621301..15624754	5.95	29.58468	255	768	3	E.R.: 4, mito: 3, nucl: 1.5, cysk_nucl: 1.5, chlo: 1, cyto: 1
MdCML32	MDP0000834180	chr9:29192629..29193093	5.05	16.87021	154	465	4	cyto: 5, chlo: 3, nucl: 3, extr: 1, cysk: 1
MdCML33	MDP0000140151	chr10:1308558..1309166	4.47	21.51673	202	609	4	nucl: 10, mito: 4
MdCML34	MDP0000259867	chr10:14642115..14650174	5.47	80.66901	709	2130	4	nucl: 7, cyto: 6
MdCML35	MDP0000859930	chr11:4014925..4016116	4.61	26.30306	227	684	4	chlo: 9, E.R.: 2, mito: 1, plas: 1
MdCML36	MDP0000135367	chr11:34230265..34232101	5.54	27.09611	244	735	3	chlo: 9.5, chlo_mito: 5.5, nucl: 3
MdCML37	MDP0000456290	chr12:7626957..7629474	4.58	43.8419	394	1185	3	chlo: 4.5, nucl: 4, chlo_mito: 3.5, cyto: 3, mito: 1.5
MdCML38	MDP0000864660	chr12:26611238..26611855	4.52	22.67528	205	618	3	mito: 7, chlo: 4, nucl: 3
MdCML39	MDP0000587060	chr13:1149146..1150223	4.96	27.9159	249	750	4	nucl: 13, cyto_nucl: 8
MdCML40	MDP0000141222	chr13:4432462..4432896	4.12	16.23301	144	435	2	chlo: 8, nucl: 2, mito: 2, extr: 2
MdCML41	MDP0000413824	chr13:20849055..20851256	3.98	20.53077	184	555	4	chlo: 8, nucl: 2, mito: 2, extr: 2
MdCML42	MDP0000148817	chr13:23750074..23753448	6.46	37.62918	331	996	2	extr: 4, vacu: 3, chlo: 2, nucl: 2, cyto: 1, mito: 1
MdCML43	MDP0000147599	chr13:31297033..31297593	4.42	20.36622	186	561	2	cyto: 9, extr: 2, pero: 2
MdCML44	MDP0000664492	chr14:7437202..7439308	6.65	27.69488	250	753	2	chlo: 10, nucl: 1, mito: 1, plas: 1
MdCML45	MDP0000178485	chr14:11244073..11246173	6.52	29.3934	270	813	2	chlo: 4, plas: 4, cyto: 2, vacu: 2, mito: 1
MdCML46	MDP0000472881	chr14:12056740..12059461	6.05	22.23064	206	621	2	nucl: 12, cyto: 1
MdCML47	MDP0000272522	chr14:15569868..15570470	5.45	21.40878	200	603	2	nucl: 14
MdCML48	MDP0000853812	chr14:22269228..22269650	4.55	15.81041	140	423	4	nucl: 6.5, cyto_nucl: 4, chlo: 3, cysk: 2, mito: 1
MdCML49	MDP0000263630	chr14:25901202..25901843	4.25	23.78951	213	642	2	chlo: 7, nucl: 2, cyto: 2, E.R.: 2
MdCML50	MDP0000602146	chr14:27094227..27094709	4.27	17.41267	160	483	4	plas: 5, nucl_plas: 4.5, mito: 3, chlo: 2, nucl: 2, cyto: 2
MdCML51	MDP0000143036	chr14:28318978..28319457	4.43	17.17411	159	480	4	chlo: 4, extr: 4, nucl: 3, cyto: 3
MdCML52	MDP0000175199	chr15:4066820..4067191	4.47	14.02187	123	372	2	cyto: 4, nucl: 2, mito: 2, extr: 2, chlo: 1, vacu: 1, E.R.: 1
MdCML53	MDP0000535637	chr15:9880713..9881372	4.67	24.39942	219	660	4	extr: 5, plas: 3, vacu: 3, golg: 2
MdCML54	MDP0000162025	chr15:20962266..20962847	4.75	21.59705	193	582	4	mito: 8, chlo: 5
MdCML55	MDP0000216112	chr15:23575377..23577654	4.76	19.27276	169	510	4	cyto: 6, nucl: 4.5, cysk_nucl: 3, mito: 2
MdCML56	MDP0000285559	chr15:26680065..26684786	6.62	56.88989	511	1536	3	mito: 5, nucl: 3, chlo: 2, plas: 2, golg: 2
MdCML57	MDP0000240032	chr15:42848527..42849000	3.59	16.95601	157	474	4	cyto: 5.5, cyto_E.R.: 3.5, chlo: 3, nucl: 2, plas: 2
MdCML58	MDP0000810260	chr17:3633514..3634101	4.68	22.25904	195	588	2	extr: 9, chlo: 3, nucl: 1

(caption on next page)

[1]GeneID number in the Genome Database for *Rosaceae* (GDR, https://www.rosaceae.org/).
[2]Chromosome number and position in which the gene resides.
3pI, theoretical isoelectric point.
4Mw, molecular weight, KDa.
5Number of amino acids of the deduced amino acid sequence.
6Length of the coding region in base pairs.
[7]Number of EF hands based on the prediction by InterProScan (https://www.ebi.ac.uk/interpro/).
[8]The prediction of protein localization in cells in WoLF PSORT II (https://www.genscript.com/wolf-psort.html?src=leftbar); Cyto, cytosol; ER, endoplasmic reticulum; Vacu, vacuolar; membrane; Chlo, chloroplast; Nucl, nuclear; Extr, extracellular; Mito, mitochondria; Cysk, cytoskeleton; Plas, plasma membrane.

Buaboocha, 2007; Munir et al., 2016) and rice (Boonburapong and Buaboocha, 2007) (Supplemental Fig. 1). Multiple sequence alignments and phylogenetic analyses revealed that CaM/CML proteins from apple, *Arabidopsis*, tomato and rice shared high levels of similarity, which suggests similar functions for those homologous members.

3.3. Conserved motifs of the MdCaMs/CMLs

The protein motif was found to be relatively conserved among CaMs but variable among CMLs. We identified the conserved motifs of MdCaMs/CMLs via the MEME program by using the following

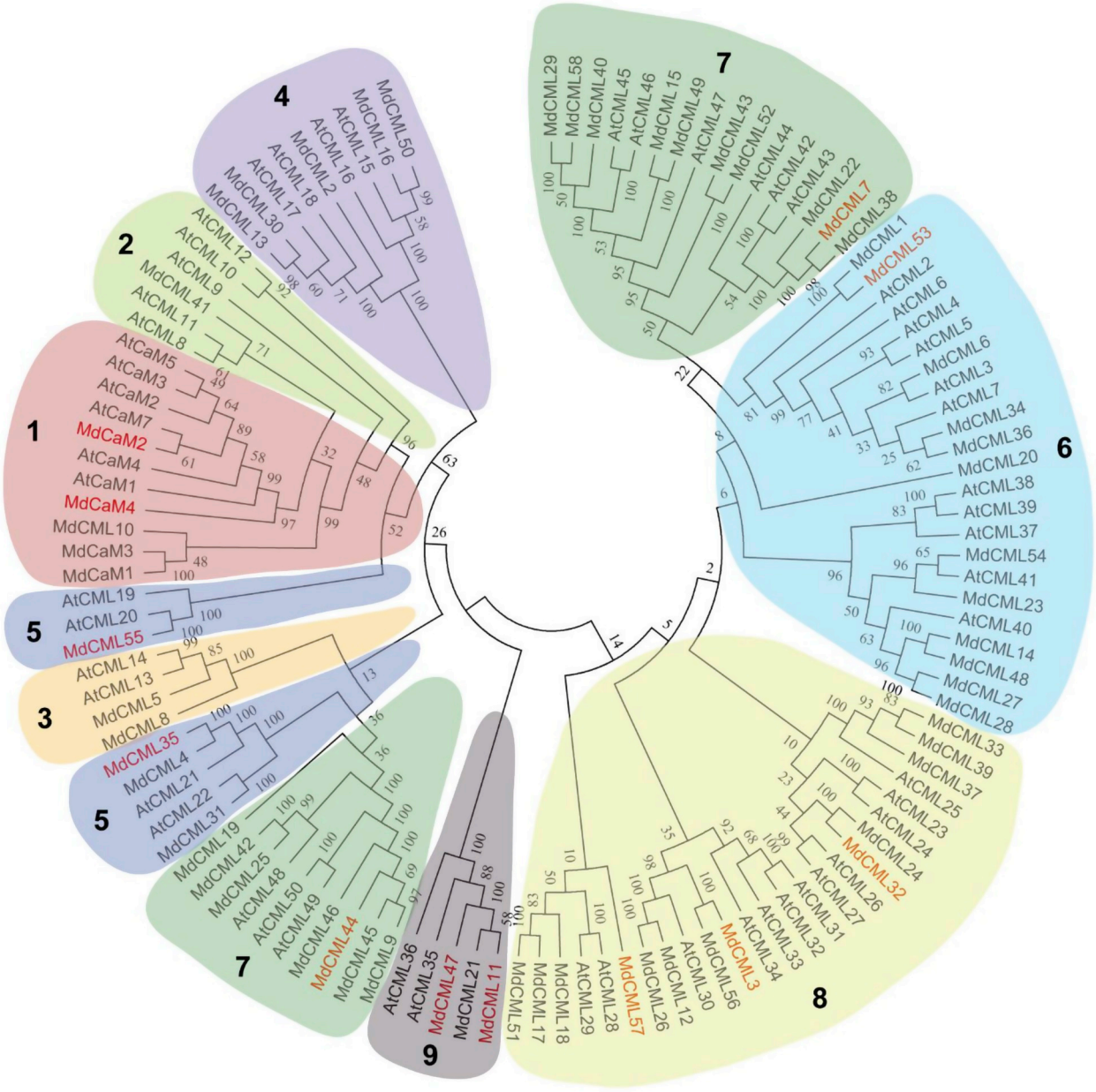

Fig. 1. Phylogenetic analysis of apple and *Arabidopsis* CaM and CML proteins. Sixty-two CaM and CML proteins from apple (4 CaMs and 58 CMLs) and 56 from *Arabidopsis* (6 CaMs and 50 CMLs) were aligned using ClustalW. The phylogenetic tree was constructed using the MEGA 6.0 program by the neighbor-joining method with bootstrap values 1000 using protein sequences. The proteins were categorized into 9 clades based on sequence similarities in amino acids between apple and *Arabidopsis*. Red color denotes the 12 selected apple genes detected by qRT-PCR in Fig. 7. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

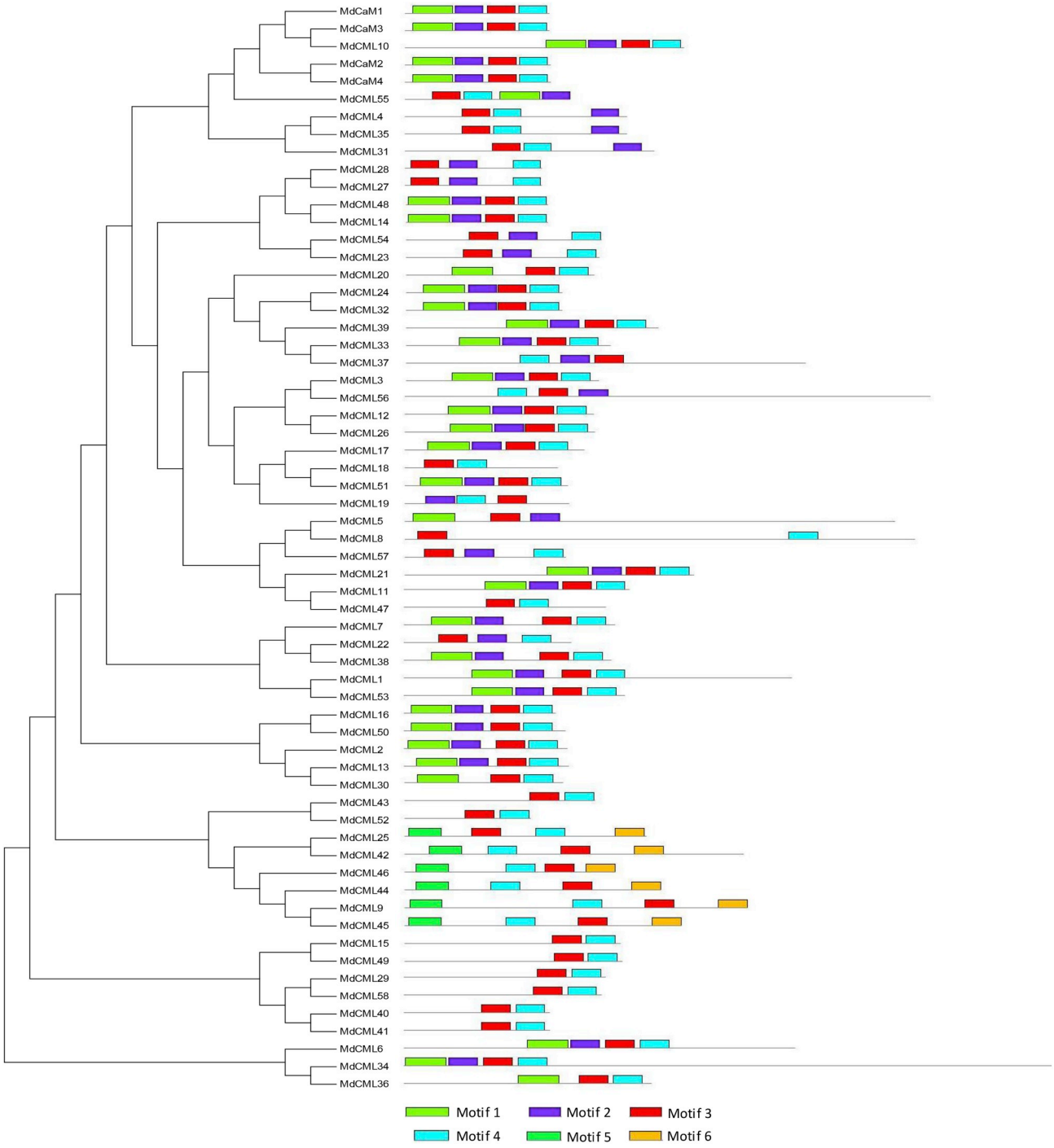

Fig. 2. Gene structure and phylogenetic relationship of *MdCaM* and *MdCML* genes. The 4 *MdCaM* and 58 MdCML genes were arranged according to the phylogenetic tree as described in Fig. 1. Six conserved motifs identified in both *CaM* and *CML* genes by the MEME program (http://meme-suite.org/) are shown in different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

parameters: a maximum of 6 motifs and an optimum motif width between 6 and 50 amino acid (Fig. 2). All of the MdCaM proteins contained the typical four EF-hands (Fig. 2), each of which had the ability to bind Ca^{2+}. However, the MdCML proteins contained two to four highly conserved EF-hand motifs. In general, members with close phylogenetic relationships had high sequence similarity and similar motifs. The presence of the same type of conserved motifs might indicate functional similarity among MdCaM/CML family members.

3.4. Chromosomal locations of the MdCaMs and MdCMLs

To determine the distribution of *MdCaMs* and *MdCMLs* genes in 17 apple chromosomes, we analyzed their chromosomal location. The *MdCaMs/CMLs* genes are spread among all of the apple chromosomes, except chromosome 16 (Fig. 3a), but the distribution appear to be unbalanced. Chromosome 14 contains the most *CML* genes (8), followed by chromosome 6, 9 and 15 with each having 6 MdCML genes. Each of the 4 *MdCaM* genes is located on chromosome 3, 7, 11 and 14. However, there is only one *MdCML* gene each on chromosome 1 and 17. (Fig. 3b). This uneven distribution of *MdCaM/CML* genes on

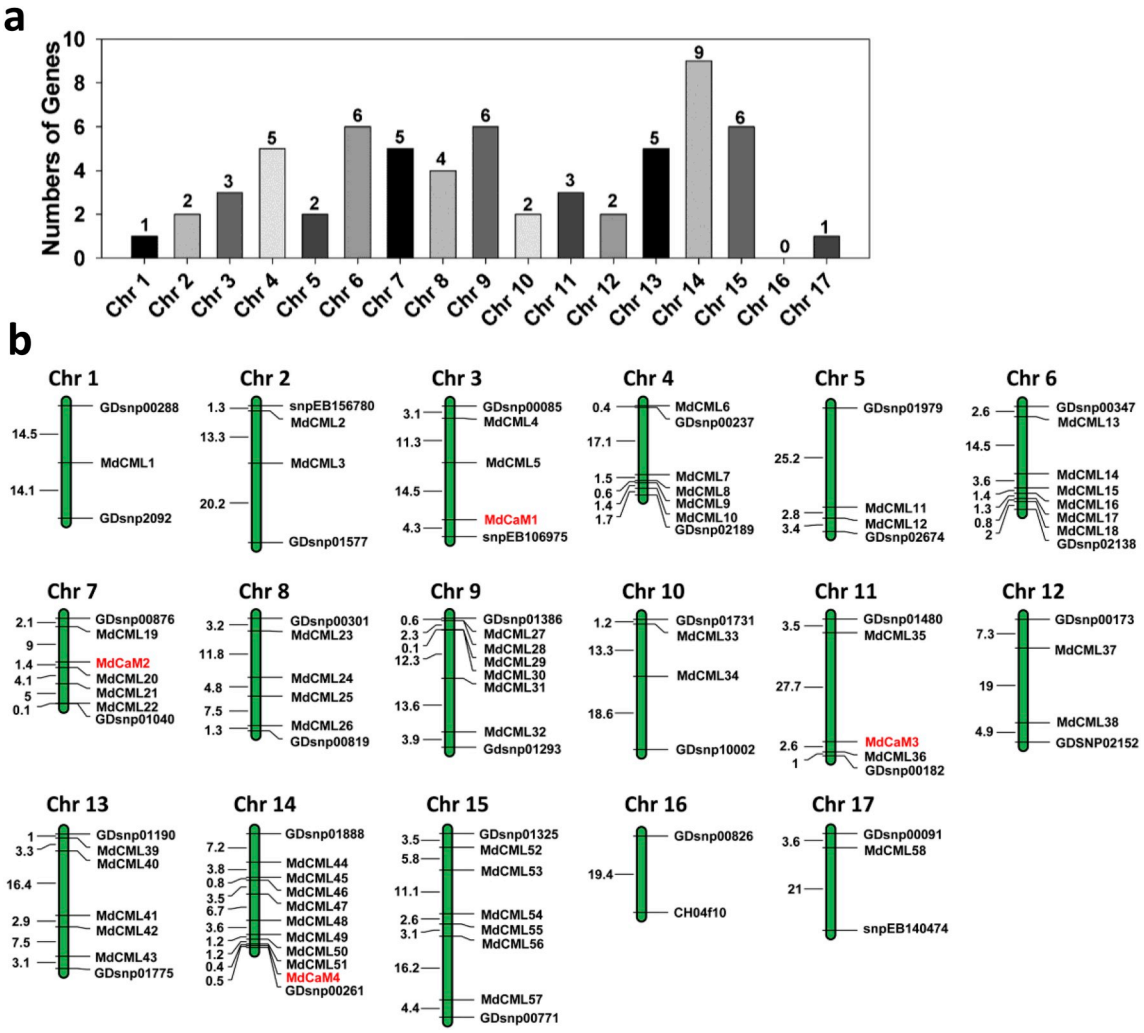

Fig. 3. The distribution of *MdCaM* and *MdCML* genes in apple chromosomes. a, The number of *MdCaM* and *MdCML* genes in each chromosome. b, *MdCaMs* and *MdCMLs* were mapped onto apple chromosomes. Names were assigned based on their location in apple chromosomes.

chromosomes indicate that genetic variations exist in the evolutionary process of the apple.

3.5. Expression profiles of MdCaM and MdCML genes in different tissues

The function of any given gene is closely related to its expression. To gain insights into the potential functions of *MdCaMs* and *MdCMLs*, we analyzed all members' transcript levels via RNA-seq to examine their expression patterns in different tissues, including roots, shoot tips, mature leaves, stamens, flowers, and mature fruit (Fig. 4). *MdCaM2* was expressed at a high level in all tissues tested, suggesting a ubiquitous role of *MdCaM2* in apple tree growth and development. In addition, *MdCML11* and *MdCML47* had similarly high expression levels in most tissues examined except fruit. Some members were expressed in specific tissues. For example, *MdCML7* and *MdCML49* had the highest expression in roots, whereas *MdCML13* and *MdCML35* showed higher expression levels in stamens compared with other parts. Interestingly, *MdCaM4*, *MdCML32*, *35*, *54* and *55* showed high expression levels in both stamens and flowers, implying the potential function of these genes in flower and fruit development. However, *MdCML14*, *15*, *16*, *18*, *20*, *27*, *30*, *37*, *50*, *52* and *58* genes showed low expression in most tissues (Fig. 4).

To investigate the function of *MdCaMs* and *MdCMLs* during apple fruit development, we analyzed their expression patterns at five fruit developmental stages using the RNA-seq database (Li et al., 2016)). The expression level of *MdCaM2* stayed very high throughout fruit development (Fig. 5). The expression level of *MdCaM4*, *MdCML11*, *12*, *17*, *21*, *32*, *47* and *57* showed developmental stage-dependent changes. As fruit developed from cell division (S1) to ripening (S5), the transcript level of *MdCML11*, *12*, *47* and *57* decreased; *MdCML21* and 32 had a high expression level only at S1 whereas *MdCML17* was highly expressed at both S1 and S5. These differential expression patterns may provide important clues for exploring their functions in fruit development in the future.

3.6. Expression patterns of MdCaM and MdCML genes in response to ABA and JA

Increasing evidence suggests that CaMs/CMLs play important roles in biotic and abiotic stress tolerance in various plant species (Reddy et al., 2011; Virdi et al., 2015; Yang et al., 2013; Zeng et al., 2015). As important stress hormones in plants, both abscisic acid (ABA) and jasmonic acid (JA) are involved in responses to various biotic and abiotic stresses (Ahmad et al., 2016; Verma et al., 2016; Vishwakarma et al., 2017). To understand potential involvement of the newly identified *MdCaM* and *MdCML* genes in responses to biotic and abiotic stresses, we analyzed the expression patterns of *MdCaM* and *MdCML* genes under ABA and JA treatments using RNA-seq (Fig. 6). In response to ABA and JA, the transcript level of *MdCML44*, *45*, and *46* was down-regulated. By contrast, the transcript level of *MdCML3*, *4* and *7* and that of

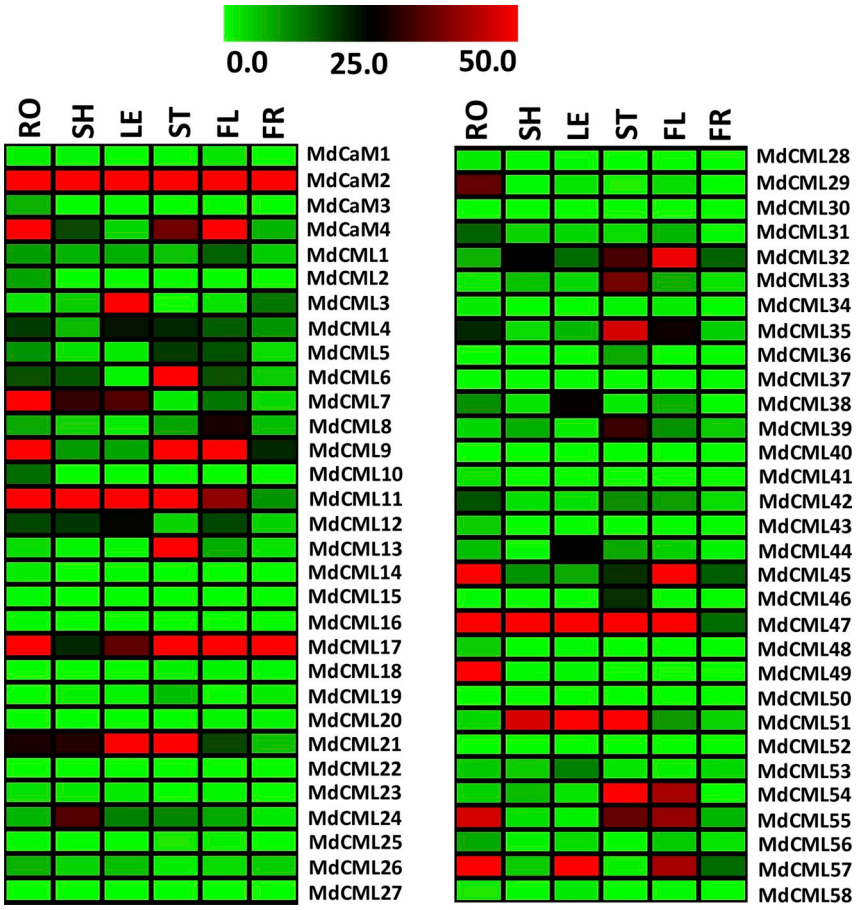

Fig. 4. Expression profiles of *MdCaM* and *MdCML* genes in different tissues of apple. The color bar represents the expression value, normalized by the reads per kilobase of exon model per million mapped reads (RPKM) algorithm. RO: Roots, SH: Shoot tips, LE: Leaves, ST: Stamens, FL: Flowers, FR: Mature fruit. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

MdCaM1 and *MdCML19* were up-regulated by ABA and JA, respectively. The results suggest that these MdCaMs/CMLs may be involved in the transduction of ABA/JA signals under various stresses.

3.7. Expression analysis of the selected genes in response to salinity and ABA by qRT-PCR

Considering that 1) high salinity is a major abiotic stress for apple tree growth and development (Duan et al., 2015; Julkowska and Testerink, 2015; Yang and Guo, 2018); 2) ABA concentration increases in response to both biotic and abiotic stresses, thereby regulating the expression of genes involved in stress adaptation (Lee and Luan, 2012; Leng et al., 2014); and 3) an increasing number of CaM/CML have been shown to be involved in salinity stress and ABA responses (Perochon et al., 2011; Reddy et al., 2011), we selected 12 candidate genes based on the subgroup classification of MdCaMs/CMLs (Fig. 1, colored in red) and their expression levels obtained in the RNA-seq data (Figs. 4 and 6) for qRT-PCR analysis of their expression patterns under salinity stress and ABA to explore the functional *MdCaMs/CMLs* members (Fig. 7). The expression level of all 12 genes was increased under salinity stress, with *CML7* transcription level being increased nearly five times in 12 h of treatment. In addition, *CML3* and 57 had the highest expression at 2 h of salinity treatment and then returned to their normal low level, whereas other genes were kept at a higher expression level from 2 to 24 h (Fig. 7). The expression level of *CML3*, *35* and *57* was increased after 2 h of ABA treatment, which is consistent with the RNA-seq result (Fig. 6). Meanwhile, *CaM2*, *CML47* and *53*'s expression was decreased in response to ABA. These results suggest that MdCaMs/CMLs family members may have diverse biological functions in response to stress conditions, such as salinity and ABA treatment.

3.8. Growth of MdCML3-overexpressing apple calli in response to salinity and ABA

The qRT-PCR result showed that the expression of *MdCML3* was increased under both NaCl and ABA treatments (Fig. 7). In addition, ABA (ABRE) and stress (TC-rich repeats) responsiveness elements were found to be present in the *MdCML3* promoter (1500 bp upstream sequence of the gene) via the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) analysis (Supplemental Table 2), which suggests that MdCML3 may play a role in adapting to ABA and/or salinity stress. To determine the function of MdCML3, we constructed a pGWB417-MdCML3 overexpression vector and transformed it into 'Orin' apple calli. Expression analysis showed significantly higher *MdCML3* transcript levels in the transgenic MdCML3-OE calli (OE-1, 2) than in the empty vector (pGWB417) control calli (417–1, 2). No difference in fresh weight was detected between MdCML3-OE and control calli (417–1, 2) under normal conditions (Fig. 8b and e), but when grown under 100 mM NaCl or 10 μM ABA, MdCML3-OE calli had significantly higher fresh weight (Fig. 8c–e), indicating that MdCML3 confers a level of tolerance to salinity stress and ABA level in apple. These results also demonstrate that gene expression analysis of *MdCaM/CMLs* can provide good clues for the evaluation of their physiological function.

4. Discussion

An increasing number of studies have shown that Ca^{2+} is an important second messenger in eliciting responses to many biotic and abiotic signals in plants (DeFalco et al., 2009; McAinsh and Pittman, 2009). Myriad Ca^{2+} binding proteins in plants that function as Ca^{2+} sensors to decode complex Ca^{2+} signatures (Boonburapong and

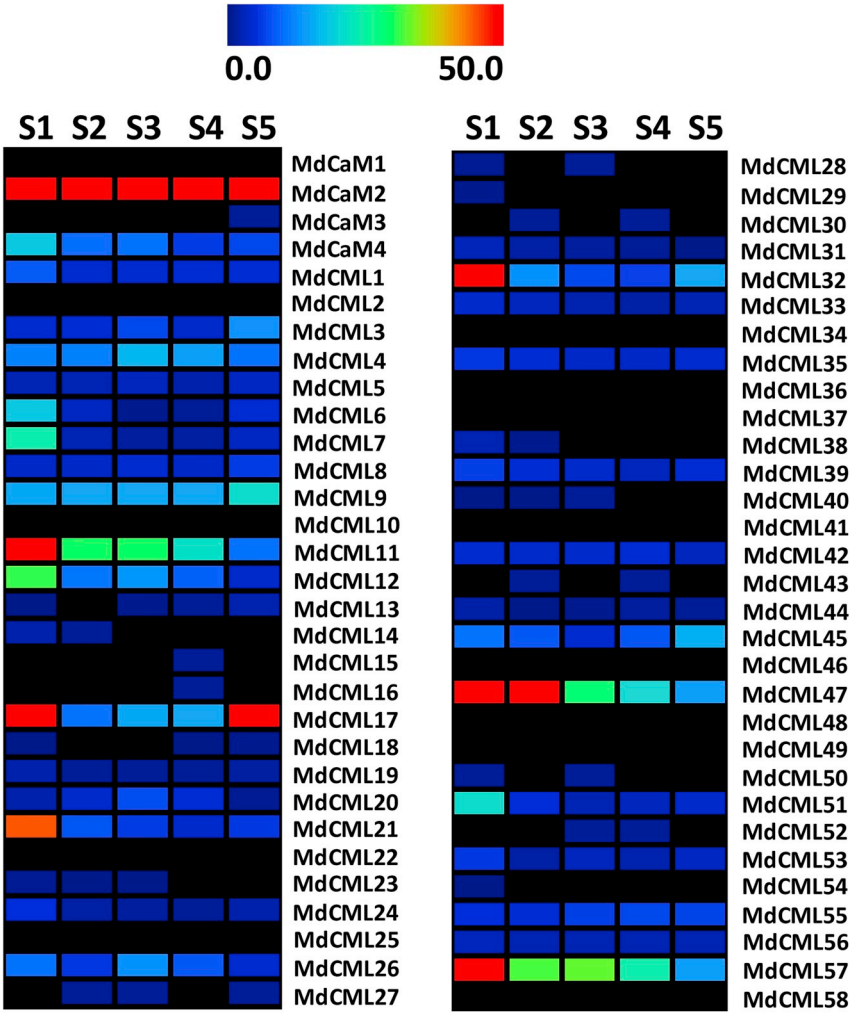

Fig. 5. Expression profiles of *MdCaM* and *MdCML* genes during apple fruit development. The color bar represents the expression value, normalized by the reads per kilobase of exon model per million mapped reads (RPKM) algorithm. 'Greensleeves' fruit samples were taken at five developmental stages, from cell division (S1) to fruit ripening (S5), with three replicates at each stage. DAB, days after full bloom. S1: 18 DAB. S2: 37 DAB. S3: 67 DAB. S4: 90 DAB. S5: 132 DAB. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Buaboocha, 2007; Day et al., 2002). These Ca^{2+} binding proteins respond to the changing cellular Ca^{2+} concentration and amplify downstream signal transduction, thereby eliciting a physiological response corresponding to the signal. Among these Ca^{2+} sensors, CaMs and CMLs are the most conserved major calcium sensor proteins in plants. CaMs are about 149 amino acid in length and carry two pairs of EF-hand motifs and share over 90% identity in amino acid sequence (Yang and Poovaiah, 2003). By following these criteria, we identified 4 *CaM* genes, with additional 58 *CaM-like (CML)* genes in the apple genome (Table 1). Apple has more CML members than *Arabidopsis* (50 CML-encoding genes), rice (32 CML-encoding genes) and tomato (52 CML-encoding genes), but less than grapevine (62 CML-encoding genes) (Boonburapong and Buaboocha, 2007; McCormack and Braam, 2003; Munir et al., 2016; Vandelle et al., 2018). In agreement with previous studies, different subcellular localizations are predicted for the MdCaMs/CMLs (Table 1), which suggests their diverse biological functions in apple. Some of the CaM/CML members are involved in calcium signal transduction and stress resistance in *Arabidopsis* or other crop plants (Ahmad et al., 2016; DeFalco et al., 2009; Leng et al., 2014; McCormack and Braam, 2003; McCormack et al., 2005; Reddy et al., 2011; Yang and Guo, 2018). The neighbor-joining phylogenetic trees constructed for MdCaM/CMLs with *Arabidopsis*, rice and tomato CaM/CMLs demonstrate their evolutionary relationships and potential similarities in function (Fig. 1, S—Fig. 1). These phylogenetic trees will be a useful reference for future studies on MdCaMs/CMLs. Moreover, the motif analysis and characterization will also help in future exploration of gene functions. The conservation and divergence of motif numbers present in the MdCaM/CML proteins (Fig. 2) are expected to lead to functional similarities or differences between various MdCaM/CML family members (Day et al., 2002; Zeng et al., 2017).

The important functions of CaMs/CMLs in plant development and stress tolerance have been widely reported. For example, CaM takes part in plant development through interaction with various transcription factors, including members of the NAC and WRKY families (Kim et al., 2007; Park et al., 2005). AtCML39 is involved in regulating seed development and seedling establishment in *Arabidopsis* (Bender et al., 2013; Midhat et al., 2018). Expression analysis demonstrated that various CMLs are also involved in response to abiotic stresses such as drought and salinity. CML18 has been shown to increase plant salinity tolerance through interaction with the Na^+/H^+ antiporter AtNHX1 on the tonoplast (Yamaguchi et al., 2005). AtCML20 is a negative regulator for plant drought resistant as *CML20* overexpression lines were hypersensitive to drought stress (Wu et al., 2017). Considering that the function of any given gene is closely related to its expression (Anil et al., 2000; Vanderbeld and Snedden, 2007), we determined the expression patterns of *MdCaMs/CMLs* in different apple tissues and in response to plant hormones (ABA and JA) through RNA-seq analysis to evaluate their potential functions. We found that the evolutionarily conserved

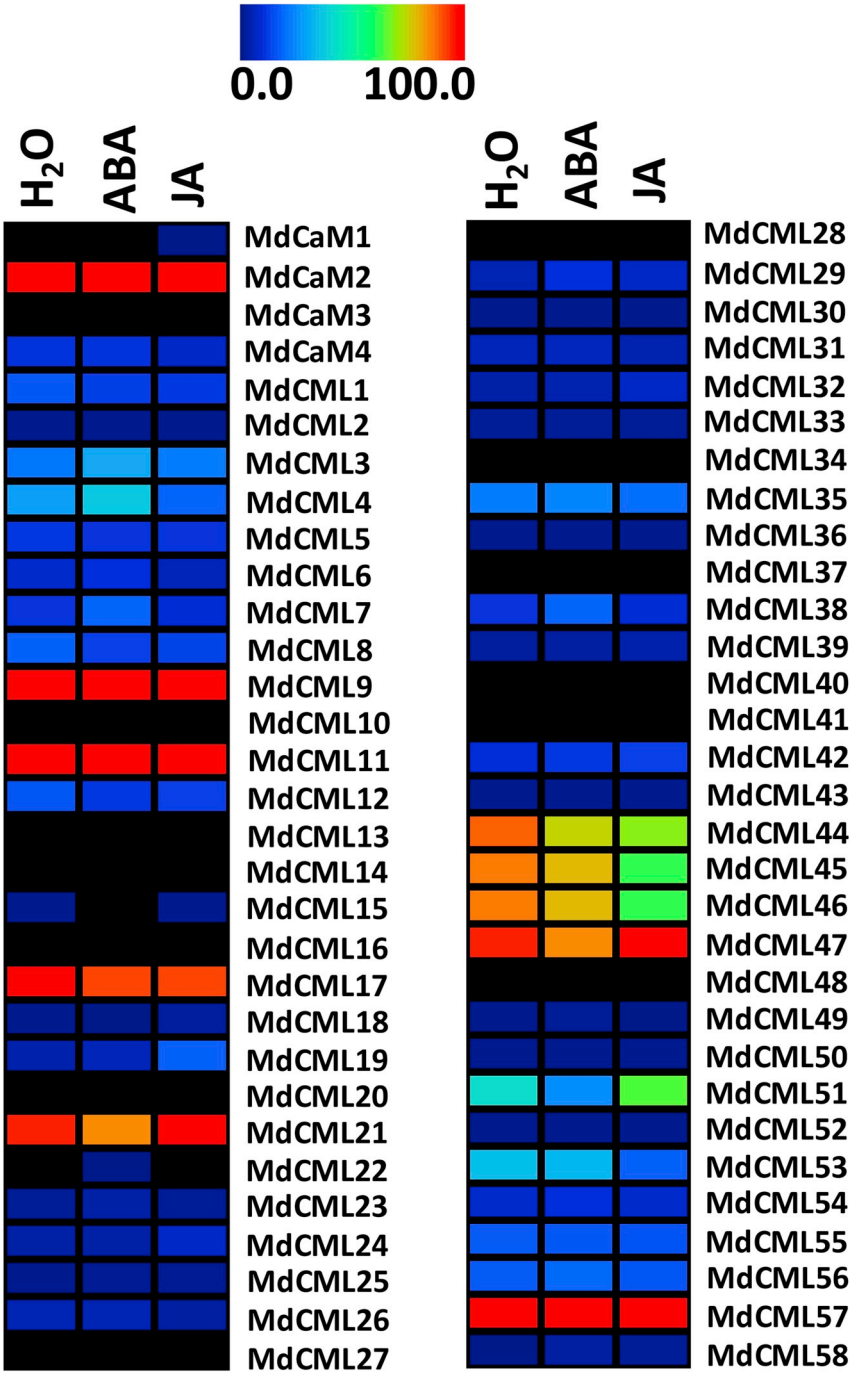

Fig. 6. Expression profiles of *MdCaM* and *MdCML* genes in response to abscisic acid (ABA) and jasmonic acid (JA) treatment. The color bar represents the expression value, normalized by the reads per kilobase of exon model per million mapped reads (RPKM) algorithm. H_2O: Control; ABA: 1 μM ABA fed via petiole; JA: 10 μM JA fed via petiole. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CaMs showed broad spatio-temporal expression profiles in various tissues and during fruit development, whereas most CMLs displayed more tissue-specific expression patterns (Figs. 4 and 5). Among the four *CaMs*, *MdCaM2* had the high expression level in all the tissues tested and under hormone treatments, suggesting a vital and universal function of *MdCaM2* in apple tree growth and fruit development. Moreover, some *MdCMLs* were highly expressed in a specific tissue, such as *CML7*, *29* and *49* in roots, *CML24* in shoot tips, *CML3* in leaf, *CML54* and *55* in both stamens and flowers. During fruit develop, *MdCMLs* showed differential expression patterns (Fig. 5). The expression level of *MdCML11*, *47* and *57* gradually decreased with fruit development, suggesting that these CML members may play a more important role in early fruit development than fruit ripening. In light of important roles of ABA and JA in plant stress responses (Ahmad et al., 2016; Lee and Luan, 2012), the expression patterns of *MdCaMs/CMLs* in response to ABA and JA may be useful to exploring the functional members for stress tolerance (Fig. 6). In addition, we also analyzed the expression of 12 selected *MdCaMs/CMLs* in response to salinity and ABA by qRT-PCR (Fig. 7). Consistent with the RNA-seq results, *MdCML3*'s expression showed a rapid increase whereas *MdCML47*'s expression decreased in response to ABA. For salinity response, most selected members had higher expression levels in 2–24 h of treatment, especially *MdCML7*'s expression increased almost 5 times in 12 h. Moreover, when *MdCML3* was overexpressed in apple calli, the transgenic calli grew significantly better

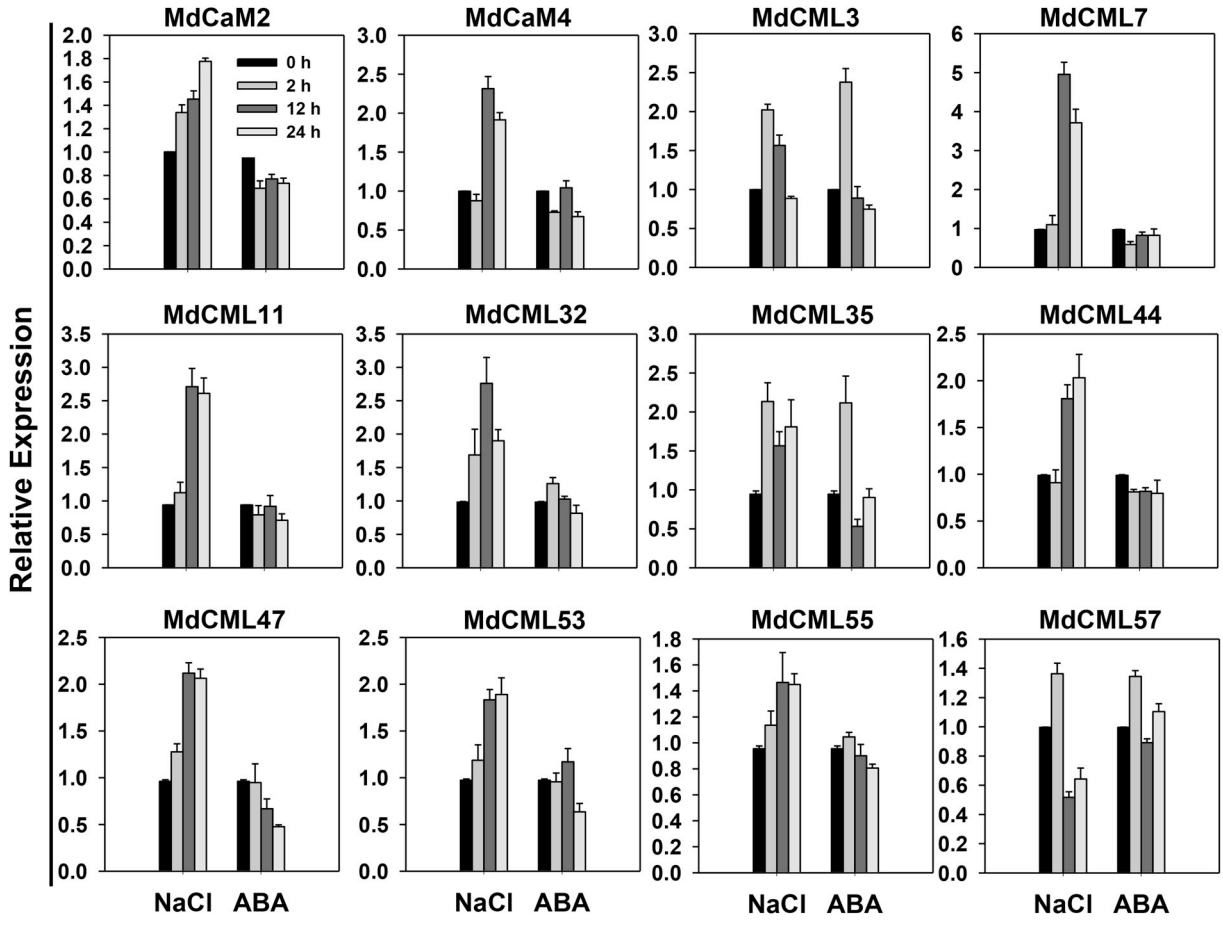

Fig. 7. The expression patterns of 12 selected *MdCaM* and *MdCML* genes in the leaves of 'Greensleeves' apple in response to NaCl and ABA treatment. Plants were grown on MS medium at 23 °C under fluorescent lights at ~50 μmol photons m^{-2} s^{-1} in a 16-hr photoperiod. The treated plants were drenched with 200 mM NaCl or 50 μM ABA, with water as control. Each treatment was replicated 5 times with 3 plants per replicate. Gene expression was detected using qRT-PCR.

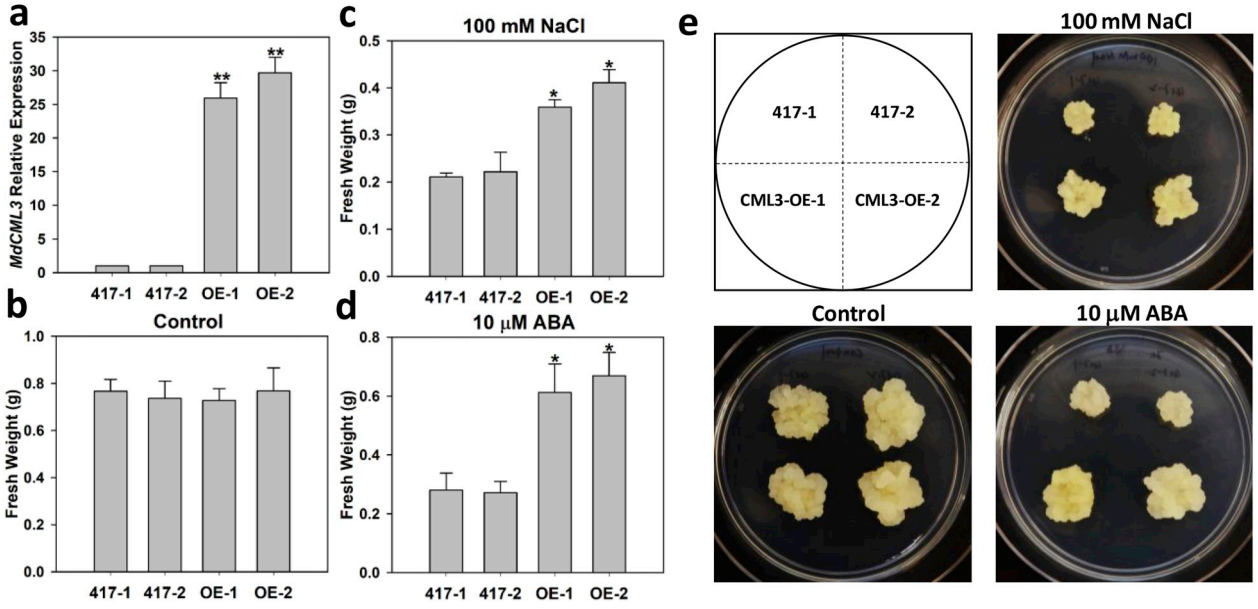

Fig. 8. Growth of *MdCML3*-overexpressing 'Orin' apple calli in response to NaCl or ABA treatment. a, qRT-PCR analysis of the expression of *MdCML3* in control (417–1, 2) and *MdCML3* overexpression (OE-1, 2) calli. b, Fresh weight of 2-week-old calli grown on control medium. c, Fresh weight of 2-week-old calli grown on 100 mM NaCl medium. d, Fresh weight of 2-week-old calli grown on 10 μM ABA medium. e, Picture of 2-week-old calli grown in control, 100 mM NaCl or 10 μM ABA medium.

under salinity and ABA conditions, suggesting that MdCML3 may play a role in the tolerance of apple trees to salinity and drought. In summary, our identification and expression analysis of *MdCaMs/CMLs* lays a foundation for future functional studies to elucidate their physiological roles in apple.

5. Conclusion

We have identified and characterized 4 MdCaM and 58 MdCML proteins containing functional EF-hand motifs in the apple genome. Expression analysis reveals that *MdCaM/CML* members have diverse expression patterns in different tissues and stress responses, and overexpression of stress-induced *MdCML3* significantly enhances the tolerance of apple calli to salinity and ABA. These findings provide new insights into the specificity of CML gene expression, and lay a foundation for further examining the function of *MdCaM/CML* genes in calcium signaling and stress responses in apple and other tree fruits in Rosaceae.

Acknowledgment

This work is supported in part by USDA National Institute of Food and Agriculture - Specialty Crop Research Initiative project “AppleRoot2Fruit: Accelerating the development, evaluation and adoption of new apple rootstocks” (2016-51181-25406). We thank Drs. Zhangjun Fei, Yang Bai and Yi Zheng at the Boyce Thompson Institute for help in the analysis of RNAseq data, Dr. Takaya Moriguchi of National Institute of Fruit Tree Science in Japan for providing ‘Orin’ apple calli, and Dr. Yongjian Chang at North American Plants for providing apple rootstocks.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.014.

Conflicts of interest

The authors declare no competing interests.

Authors' contributions

CL, DM and LC designed the research. CL, DM and JZ performed the research. CL and DM performed bioinformatics analysis, including gene identification and RNA-seq data analysis. JZ performed qRT-PCR. CL, DM and LC wrote the article.

References

Ahmad, P., Rasool, S., Gul, A., Sheikh, S.A., Akram, N.A., Ashraf, M., Kazi, A.M., Gucel, S., 2016. Jasmonates: multifunctional roles in stress tolerance. Front. Plant Sci. 7, 813.
An, X.H., Tian, Y., Chen, K.Q., Wang, X.F., Hao, Y.J., 2012. The apple WD40 protein MdTTG1 interacts with bHLH but not MYB proteins to regulate anthocyanin accumulation. J. Plant Physiol. 169, 710–717.
Anil, V.S., Harmon, A.C., Rao, K.S., 2000. Spatio-temporal accumulation and activity of calcium-dependent protein kinases during embryogenesis, seed development, and germination in sandalwood. Plant Physiol. 122, 1035–1043.
Bailey, T.L., Elkan, C., 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In: Proceedings. International Conference on Intelligent Systems for Molecular Biology 2. pp. 28–36.
Batistic, O., Kudla, J., 2012. Analysis of calcium signaling pathways in plants. Biochim. Biophys. Acta 1820, 1283–1293.
Bender, K.W., Rosenbaum, D.M., Vanderbeld, B., Ubaid, M., Snedden, W.A., 2013. The Arabidopsis calmodulin-like protein, CML39, functions during early seedling establishment. Plant J. : Cell Mol. Biol. 76, 634–647.
Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. 1, 11–21.
Boonburapong, 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., Duanmu, 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, e0141888.
Cheng, H.Q., Han, L.B., Yang, C.L., Wu, X.M., Zhong, N.Q., Wu, J.H., Wang, F.X., Wang, H.Y., Xia, G.X., 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, 1935–1950.
Cheng, L., Zhou, R., Reidel, E.J., Sharkey, T.D., Dandekar, A.M., 2005. Antisense inhibition of sorbitol synthesis leads to up-regulation of starch synthesis without altering CO 2 assimilation in apple leaves. Planta 220, 767–776.
Cheng, S.H., Willmann, M.R., Chen, H.C., Sheen, J., 2002. Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. Plant Physiol. 129, 469–485.
Cornille, A., Giraud, T., Smulders, M.J., Roldan-Ruiz, I., Gladieux, P., 2014. The domestication and evolutionary ecology of apples. Trends Genet. : TIG (Trends Genet.) 30, 57–65.
Day, I.S., Reddy, V.S., Shad Ali, G., Reddy, A.S., 2002. Analysis of EF-hand-containing proteins in arabidopsis. Genome Biol. 3 RESEARCH0056.
de Freitas, S.T., Mitcham, E.I., 2012. 3 factors involved in fruit calcium deficiency disorders. Hortic. Rev. 40, 107–146.
DeFalco, T.A., Bender, K.W., Snedden, W.A., 2009. Breaking the code: Ca2+ sensors in plant signalling. Biochem. J. 425, 27–40.
Dodd, A.N., Kudla, J., Sanders, D., 2010. The language of calcium signaling. Annu. Rev. Plant Biol. 61, 593–620.
Duan, L., Sebastian, J., Dinneny, J.R., 2015. Salt-stress regulation of root system growth and architecture in Arabidopsis seedlings. Methods Mol. Biol. 1242, 105–122.
Duan, N., Bai, Y., Sun, H., Wang, N., Ma, Y., Li, M., Wang, X., Jiao, C., Legall, N., Mao, L., Wan, S., Wang, K., He, T., Feng, S., Zhang, Z., Mao, Z., Shen, X., Chen, X., Jiang, Y., Wu, S., Yin, C., Ge, S., Yang, L., Jiang, S., Xu, H., Liu, J., Wang, D., Qu, C., Wang, Y., Zuo, W., Xiang, L., Liu, C., Zhang, D., Gao, Y., Xu, Y., Xu, K., Chao, T., Fazio, G., Shu, H., Zhong, G.Y., Cheng, L., Fei, Z., 2017. Genome re-sequencing reveals the history of apple and supports a two-stage model for fruit enlargement. Nat. Commun. 8, 249.
Ferguson, I., Watkins, C., 1989. Bitter pit in apple fruit. Hortic. Rev. 11, 289–355.
Gifford, J.L., Walsh, M.P., Vogel, H.J., 2007. Structures and metal-ion-binding properties of the Ca2+-binding helix-loop-helix EF-hand motifs. Biochem. J. 405, 199–221.
Grabarek, Z., 2006. Structural basis for diversity of the EF-hand calcium-binding proteins. J. Mol. Biol. 359, 509–525.
Hashimoto, K., Kudla, J., 2011. Calcium decoding mechanisms in plants. Biochimie 93, 2054–2059.
Hetherington, A.M., Brownlee, C., 2004. The generation of Ca(2+) signals in plants. Annu. Rev. Plant Biol. 55, 401–427.
Julkowska, M.M., Testerink, C., 2015. Tuning plant signaling and growth to survive salt. Trends Plant Sci. 20, 586–594.
Kanchiswamy, C.N., Mohanta, T.K., Capuzzo, A., Occhipinti, A., Verrillo, F., Maffei, M.E., Malnoy, M., 2013. Differential expression of CPKs and cytosolic Ca2+ variation in resistant and susceptible apple cultivars (Malus x domestica) in response to the pathogen Erwinia amylovora and mechanical wounding. BMC Genomics 14, 760.
Kim, H.S., Park, B.O., Yoo, J.H., Jung, M.S., Lee, S.M., Han, H.J., Kim, K.E., Kim, S.H., Lim, C.O., Yun, D.J., Lee, S.Y., Chung, W.S., 2007. Identification of a calmodulin-binding NAC protein as a transcriptional repressor in Arabidopsis. J. Biol. Chem. 282, 36292–36302.
Leba, L.J., Cheval, C., Ortiz-Martin, I., Ranty, B., Beuzon, C.R., Galaud, J.P., Aldon, D., 2012. CML9, an Arabidopsis calmodulin-like protein, contributes to plant innate immunity through a flagellin-dependent signalling pathway. Plant J. : Cell Mol. Biol. 71, 976–989.
Lee, S.C., Luan, S., 2012. ABA signal transduction at the crossroad of biotic and abiotic stress responses. Plant Cell Environ. 35, 53–60.
Leng, P., Yuan, B., Guo, Y., 2014. The role of abscisic acid in fruit ripening and responses to abiotic stress. J. Exp. Bot. 65, 4577–4588.
Lewit-Bentley, A., Rety, S., 2000. EF-hand calcium-binding proteins. Curr. Opin. Struct. Biol. 10, 637–643.
Li, M., Li, D., Feng, F., Zhang, S., Ma, F., Cheng, L., 2016. Proteomic analysis reveals dynamic regulation of fruit development and sugar and acid accumulation in apple. J. Exp. Bot. 67, 5145–5157.
Li, M., Li, P., Ma, F., Dandekar, A.M., Cheng, L., 2018. Sugar metabolism and accumulation in the fruit of transgenic apple trees with decreased sorbitol synthesis. Horticul. Res. 5, 60.
Liao, J., Deng, J., Qin, Z., Tang, J., Shu, M., Ding, C., Liu, J., Hu, C., Yuan, M., Huang, Y., Yang, R., Zhou, Y., 2017. Genome-wide identification and analyses of calmodulins and calmodulin-like proteins in Lotus japonicas. Front. Plant Sci. 8, 482.
Luan, S., 2009. The CBL-CIPK network in plant calcium signaling. Trends Plant Sci. 14, 37–42.
Magnan, F., Ranty, B., Charpenteau, M., Sotta, B., Galaud, J.P., Aldon, D., 2008. Mutations in AtCML9, a calmodulin-like protein from Arabidopsis thaliana, alter plant responses to abiotic stress and abscisic acid. Plant J. : Cell Mol. Biol. 56, 575–589.
McAinsh, M.R., Pittman, J.K., 2009. Shaping the calcium signature. New Phytol. 181, 275–294.
McCormack, E., Braam, J., 2003. Calmodulins and related potential calcium sensors of Arabidopsis. New Phytol. 159, 585–598.
McCormack, E., Tsai, Y.C., Braam, J., 2005. Handling calcium signaling: arabidopsis CaMs and CMLs. Trends Plant Sci. 10, 383–389.
Meng, D., Gu, Z., Li, W., Wang, A., Yuan, H., Yang, Q., Li, T., 2014. Apple MdABCF assists in the transportation of S-RNase into pollen tubes. Plant J.: Cell Mol. Biol. 78, 990–1002.
Meng, D., He, M., Bai, Y., Xu, H., Dandekar, A.M., Fei, Z., Cheng, L., 2018a. Decreased sorbitol synthesis leads to abnormal stamen development and reduced pollen tube

growth via an MYB transcription factor, MdMYB39L, in apple (Malus domestica). New Phytol. 217, 641–656.
Meng, D., Li, C., Park, H.-J., González, J., Wang, J., Dandekar, A.M., Turgeon, B.G., Cheng, L., 2018b. Sorbitol modulates resistance to Alternaria alternata by regulating the expression of an NLR resistance gene in apple. Plant Cell 30, 1562–1581.
Midhat, U., Ting, M.K.Y., Teresinski, H.J., Snedden, W.A., 2018. The calmodulin-like protein, CML39, is involved in regulating seed development, germination, and fruit development in Arabidopsis. Plant Mol. Biol 96, 375–392.
Mohanta, T.K., Kumar, P., Bae, H., 2017. Genomics and evolutionary aspect of calcium signaling event in calmodulin and calmodulin-like proteins in plants. BMC Plant Biol. 17, 38.
Munir, S., Khan, M.R., Song, J., 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. : PPB (Plant Physiol. Biochem.) 102, 167–179.
Nie, S., Zhang, M., Zhang, L., 2017. Genome-wide identification and expression analysis of calmodulin-like (CML) genes in Chinese cabbage (Brassica rapa L. ssp. pekinensis). BMC Genomics 18, 842.
Ozgur, R., Turkan, I., Uzilday, B., Sekmen, A.H., 2014. Endoplasmic reticulum stress triggers ROS signalling, changes the redox state, and regulates the antioxidant defence of Arabidopsis thaliana. J. Exp. Bot. 65, 1377–1390.
Park, C.Y., Lee, J.H., Yoo, J.H., Moon, B.C., Choi, M.S., Kang, Y.H., Lee, S.M., Kim, H.S., Kang, K.Y., Chung, W.S., Lim, C.O., Cho, M.J., 2005. WRKY group IId transcription factors interact with calmodulin. FEBS Lett. 579, 1545–1550.
Perochon, A., Aldon, D., Galaud, J.P., Ranty, B., 2011. Calmodulin and calmodulin-like proteins in plant calcium signaling. Biochimie 93, 2048–2053.
Ranty, B., Aldon, D., Cotelle, V., Galaud, J.P., Thuleau, P., Mazars, C., 2016. Calcium sensors as key hubs in plant responses to biotic and abiotic stresses. Front. Plant Sci. 7, 327.
Reddy, A.S., Ali, G.S., Celesnik, H., Day, I.S., 2011. Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. Plant Cell 23, 2010–2032.
Suzuki, N., Koussevitzky, S., Mittler, R., Miller, G., 2012. ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ. 35, 259–270.
Takabatake, R., Karita, E., Seo, S., Mitsuhara, I., Kuchitsu, K., Ohashi, Y., 2007. Pathogen-induced calmodulin isoforms in basal resistance against bacterial and fungal pathogens in tobacco. Plant Cell Physiol. 48, 414–423.
Vandelle, E., Vannozzi, A., Wong, D., Danzi, D., Digby, A.M., 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. : PPB (Plant Physiol. Biochem.) 129, 221–237.
Vanderbeld, B., Snedden, W.A., 2007. Developmental and stimulus-induced expression patterns of Arabidopsis calmodulin-like genes CML37, CML38 and CML39. Plant Mol. Biol. 64, 683–697.
Verma, V., Ravindran, P., Kumar, P.P., 2016. Plant hormone-mediated regulation of stress responses. BMC Plant Biol. 16, 86.
Virdi, A.S., Singh, S., Singh, P., 2015. Abiotic stress responses in plants: roles of calmodulin-regulated proteins. Front. Plant Sci. 6, 809.
Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R.K., Kumar, V., Verma, R., Upadhyay, R.G., Pandey, M., Sharma, S., 2017. Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Front. Plant Sci. 8, 161.
Wang, S.S., Diao, W.Z., Yang, X., Qiao, Z., Wang, M., Acharya, B.R., 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, 2372–2386.
Wei, M., Wang, S., Dong, H., Cai, B., Tao, J., 2016. Characterization and comparison of the CPK gene family in the apple (malus x domestica) and other Rosaceae species and its response to Alternaria alternata infection. PLoS One 11, e0155590.
Weinl, S., Kudla, J., 2009. The CBL-CIPK Ca(2+)-decoding signaling network: function and perspectives. New Phytol. 184, 517–528.
Wu, T., Wang, Y., Zheng, Y., Fei, Z., Dandekar, A.M., Xu, K., Han, Z., Cheng, L., 2015. Suppressing sorbitol synthesis substantially alters the global expression profile of stress response genes in apple (malus domestica) leaves. Plant Cell Physiol. 56, 1748–1761.
Wu, X., Qiao, Z., Liu, H., Acharya, B.R., 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, G.Y., Rocha, P.S., Wang, M.L., Xu, M.L., Cui, Y.C., Li, L.Y., Zhu, Y.X., Xia, X., 2011. A novel rice calmodulin-like gene, OsMSR2, enhances drought and salt tolerance and increases ABA sensitivity in Arabidopsis. Planta 234, 47–59.
Yamaguchi, T., Aharon, G.S., Sottosanto, J.B., Blumwald, E., 2005. Vacuolar Na+/H+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca2+- and pH-dependent manner. Proc. Natl. Acad. Sci. U.S.A. 102, 16107–16112.
Yang, T., Peng, H., Whitaker, B.D., Jurick, W.M., 2013. Differential expression of calcium/calmodulin-regulated SlSRs in response to abiotic and biotic stresses in tomato fruit. Physiol. Plantarum 148, 445–455.
Yang, T., Poovaiah, B.W., 2003. Calcium/calmodulin-mediated signal network in plants. Trends Plant Sci. 8, 505–512.
Yang, X., Wang, S.S., Wang, M., Qiao, Z., Bao, C.C., 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, 225–236.
Yang, Y., Guo, Y., 2018. Elucidating the molecular mechanisms mediating plant salt-stress responses. New Phytol. 217, 523–539.
Yoo, J.H., Park, C.Y., Kim, J.C., Heo, W.D., Cheong, M.S., Park, H.C., Kim, M.C., Moon, B.C., Choi, M.S., Kang, Y.H., Lee, J.H., Kim, H.S., Lee, S.M., Yoon, H.W., Lim, C.O., Yun, D.J., Lee, S.Y., Chung, W.S., Cho, M.J., 2005. Direct interaction of a divergent CaM isoform and the transcription factor, MYB2, enhances salt tolerance in arabidopsis. J. Biol. Chem. 280, 3697–3706.
Zeng, H., Xu, L., Singh, A., Wang, H., Du, L., Poovaiah, B.W., 2015. Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses. Front. Plant Sci. 6, 600.
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.
Zhang, K., Yue, D., Wei, W., Hu, Y., Feng, J., Zou, Z., 2016. Characterization and functional analysis of calmodulin and calmodulin-like genes in fragaria vesca. Front. Plant Sci. 7, 1820.
Zhao, Y., Liu, W., Xu, Y.P., Cao, J.Y., Braam, J., Cai, X.Z., 2013. Genome-wide identification and functional analyses of calmodulin genes in Solanaceous species. BMC Plant Biol. 13, 70.
Zhu, X., Dunand, C., Snedden, W., Galaud, J.P., 2015. CaM and CML emergence in the green lineage. Trends Plant Sci. 20, 483–489.
Zhu, X., Robe, E., Jomat, L., Aldon, D., Mazars, C., Galaud, J.P., 2017. CML8, an arabidopsis calmodulin-like protein, plays a role in Pseudomonas syringae plant immunity. Plant Cell Physiol. 58, 307–319.