

Genome-wide identification and expression profiling of the calmodulin-binding transcription activator gene family in apple (*Malus × domestica*) under biotic and abiotic stress conditions

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Genome-wide identification and expression profiling of the calmodulin-binding transcription activator gene family in apple (*Malus × domestica*) under biotic and abiotic stress conditions

Abstract

Calmodulin-binding transcription activators (CAMTAs) are pivotal regulators that integrate calcium signaling with plant developmental and stress-response programs. This study identified eight *MdCAMTA* genes in the apple (*Malus domestica*) genome, which encode 13 isoforms through alternative splicing. Phylogenetic analysis indicated that *MdCAMTA* proteins cluster into two ancient clades, with a divergence predating the speciation of dicots. Promoter analysis identified a complex cis-regulatory landscape dominated by light-responsive elements, binding sites for MYB, MYC, and WRKY factors, and various phytohormone-responsive motifs. Transcriptomic profiling revealed pronounced tissue specificity, particularly in pollen, where a unique reciprocal expression pattern suggests stringent spatial control of CAMTA signaling. Furthermore, *MdCAMTA* genes exhibited highly specific responses to environmental challenges: *MdCAMTA1.2* emerged as a potential marker for hypoxic adaptation and viral response, while *MdCAMTA5.2* is a candidate regulator for nutrient homeostasis and fungal defense. These findings establish a foundational genomic framework for understanding how *MdCAMTA* genes coordinate apple resilience through multifaceted hormonal and environmental signaling.

Introduction

Calcium ions (Ca^{2+}) act as a pivotal secondary messenger in eukaryotes, orchestrating intracellular signaling and gene expression dynamics (Dodd et al. 2010). To perceive stress-induced calcium fluctuations, plants utilize an array of calcium sensor proteins, most notably calmodulin (CaM) (Hashimoto and Kudla 2011). Binding of Ca^{2+} triggers conformational shifts in CaM, enabling it to interact with and modulate downstream transcription factors such as the calmodulin-binding transcription activator (CAMTA) family (Reddy 2001). These activators bridge calcium signaling with diverse metabolic and stress-response pathways (Yang and Poovaiah 2002). Structural analysis shows that CAMTAs are highly conserved, characteristically containing three major domains: a CG-1 DNA-binding domain (da Costa e Silva 1994), a TIG (transcription factor immunoglobulin) domain involved in non-specific DNA interactions (Breedon and Nasmyth 1987), and a tandem repeat IQ motif (IQXXRGXXX). The latter forms a Ca^{2+} -independent calmodulin-binding region that directly captures calmodulin or calmodulin-like proteins (Bouché et al. 2002; Teresinski et al. 2023).

By recognizing and binding to specific promoter cis-elements, specifically (A/C)CGCG(C/G/T) and (A/C)CGTGT, CAMTAs modulate the transcription of their target genes (Du et al. 2009; Kaplan et al. 2006; Walley et al. 2007). Currently, CAMTA families have been characterized across diverse plant taxa, such as *Arabidopsis* (*Arabidopsis thaliana*) (Yang and Poovaiah 2002), maize (*Zea mays*) (Yue et al. 2015), grapevine (*Vitis vinifera*) (Shangguan et al. 2014), tomato (*Solanum lycopersicum*) (Yang et al. 2012), banana (*Musa* spp.), alfalfa (*Medicago sativa*) (Yang et al. 2015), citrus (Zhang et al. 2019), wheat (*Triticum aestivum*) (Yang et al. 2020), and *Phoebe bournei* (Zheng et al. 2024). These genes have been

shown to mediate developmental processes, hormonal regulation, and adaptive responses to various abiotic stresses (Finkler et al. 2007; Sedgwick and Smerdon 1999; Yang et al. 2020; Wang et al. 2013).

The cultivated apple (*Malus domestica*) is a widely distributed fruit crop in temperate regions. Its yield potential heavily depends on the tree's capacity to withstand fluctuating environmental conditions, ranging from soil characteristics and water availability to light intensity. Investigating and identifying the genes that govern both standard ontogenetic processes and stress responses can significantly deepen our understanding of the molecular mechanisms underlying the adaptive plasticity of this species. In this study, we comprehensively characterized the CAMTA transcription factor family. Utilizing a suite of bioinformatic approaches, we identified these genes within the genome of the 'Golden Delicious' apple cultivar, examined the structural organization of their encoded proteins, and mapped their promoter cis-regulatory landscapes. Furthermore, we elucidated the phylogenetic relationships of these genes with counterparts from other plant taxa, profiled their tissue-specific expression patterns, and evaluated their transcriptional dynamics under diverse biotic and abiotic challenges.

Materials and Methods

Identification of *MdCAMTA* Genes in the Apple Genome

Nucleotide and predicted protein sequences of apple (*Malus domestica*, cv. 'Golden Delicious') were retrieved from the Genome Database for Rosaceae (GDR; rosaceae.org), while chromosomal sequences were downloaded from the NCBI GenBank database. Hidden Markov Model (HMM) profiles for the characteristic domains of calmodulin-binding transcription activators—specifically CG-1 (PF03859.15), IQ (PF00612.26), and TIG (PF01833.23)—were obtained from the Pfam database (El-Gebali et al. 2018). These HMM profiles were utilized to screen the predicted apple protein database using HMMER3 (hmmer.org). To validate the retrieved candidates, all potential hits were subjected to domain architecture analysis using the SMART tool (embl-heidelberg.de) (Letunic et al. 2020). Sequences simultaneously harboring the CG-1 domain, IQ motifs, and ankyrin repeats were formally considered candidate calmodulin-binding transcription activators.

Physicochemical Characterization and Subcellular Localization of *MdCAMTA* Proteins

The molecular weight and theoretical isoelectric point (pI) of the identified apple calmodulin-binding transcription activators were calculated using the Compute pI/Mw tool on the ExPASy server (<https://www.expasy.org/>) (Gasteiger et al. 2003). The subcellular localization of these proteins was predicted using the Localizer 1.0.4 software (Sperschneider et al. 2017).

Analysis of Promoter Cis-Regulatory Elements

To investigate the transcriptional regulatory architecture of the *MdCAMTA* genes, 2000 bp genomic sequences upstream of the translation initiation codon (ATG) were extracted for each gene. Putative cis-regulatory elements within these promoter regions were subsequently identified using the PlantCARE database (Lescot et al. 2002).

Phylogenetic Analysis and Tree Visualization

Multiple sequence alignment of the CAMTA proteins was performed using the COBALT algorithm (Papadopoulos and Agarwala 2007). Phylogenetic reconstruction was subsequently carried out via the maximum likelihood (ML) method implemented in IQ-TREE (Trifinopoulos et al. 2016), deployed on the Los Alamos Web Server (<https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html>). The LG amino acid substitution model (Le and Gascuel 2008) with FreeRate heterogeneity (Soubrier et al. 2012) was determined as the optimal evolutionary model using ModelFinder (Kalyaanamoorthy et al. 2017). Branch support was evaluated with 1,000 replicates of the Ultrafast Bootstrap (UFBoot) approximation (Hoang et al. 2018). The resulting phylogenetic tree topology was visualized and annotated using the Interactive Tree Of Life (iTOL) v. 7.6 web server (Letunic and Bork 2024).

Gene Structure and Promoter Cis-Regulatory Element Analysis

The exon-intron architecture of the identified genes was generated using the Gene Structure Display Server v.2.0 (GSDS v.2.0) (Hu et al. 2014). To investigate the transcriptional regulatory landscape, 2000 bp genomic sequences upstream of the translation initiation codon were extracted for each gene. Putative cis-regulatory elements within these promoter regions were subsequently identified using the PlantCARE database (Lescot et al. 2002).

Transcriptome Data Analysis and Heatmap Visualization

To analyze gene expression, transcript abundance was quantified from RNA-Seq raw reads using Salmon (v.1.10.0) with the quasi-mapping algorithm and automatic library type detection (-l A) (Patro et al. 2017). The CDS sequences of *MdCAMTA* genes served as the reference transcriptome for index construction. Downstream data analysis, including heatmap construction and hierarchical clustering, was performed in Python using the Pandas and Seaborn libraries (Waskom 2021; McKinney 2010). To stabilize variance, TPM (Transcripts Per Million) values were transformed using a $\log_2(X+1)$ function. Hierarchical clustering was subsequently executed using the Ward linkage method and Euclidean distance for both rows (genes) and columns (tissues/samples). To enhance the visualization of expression patterns across tissues, the data were row-normalized using a 0 to 1 scale (standard_scale=0). The resulting dendrograms reflect the similarity in expression profiles between individual genes and biological samples. The raw sequencing data used in this study were retrieved from the NCBI Sequence Read Archive (SRA) database (Leinonen et al. 2011). The complete list of analyzed samples, including their BioProject, treatment, and reference article, is provided in Table 2.

Table 2. Transcriptome data used for expression analyses.

Group / Treatment	Cultivar	Tissue	BioProject	Reference Article
10 apple tissues (pollen, anthers, filaments, young leaves, petals, styles, stigmas, sepals, ovaries, receptacles)	Golden Delicious	pollen, anthers, filaments, young leaves, petals, styles, stigmas, sepals, ovaries, receptacles	PRJNA419119	(Pratas et al. 2018)
Sunburn	Honeycrisp	Fruit	PRJNA858858	(Waite et al. 2023)
Hypobaric conditions	Granny Smith	Fruit	PRJNA293208	(Cukrov et al. 2016)
Iron and phosphorus deficiencies	M9	Root	PRJNA505541	(Valentinuzzi et al. 2019)
<i>Alternaria alternata</i> infection	Starking Delicious	Leaf	PRJNA349086	(Zhu et al. 2017)
ASGV infection	Fuji	Apple plantlets	PRJNA233486	(Chen et al. 2014)

Results

In Silico Identification, Structural Analysis, and Chromosomal Distribution of *MdCAMTA* Genes

A total of eight *MdCAMTA* genes were identified in the genome of the 'Golden Delicious' apple cultivar. Each gene encodes a protein harboring one CG-1 domain, one TIG domain, 1–2 ankyrin repeats, and 2–3 IQ domains, which strictly conforms to the characteristic architecture of calmodulin-binding transcription activators. These genes were designated as *MdCAMTA1* through *MdCAMTA8* based on their chronological order of chromosomal localization. Their physicochemical and structural characteristics are summarized in Table 1.

Table 1. Characteristics and genomic localization of *MdCAMTA* genes in apple (*Malus domestica*).

Gene name	Locus ID	Number of isoforms	Protein length (aa)	Subcellular localization	Chromosomal location
<i>MdCAMTA1</i>	MD01G1031100	3	1091–1107	Nucleus	Chr01
<i>MdCAMTA2</i>	MD05G1044600	1	1072	Nucleus	Chr05
<i>MdCAMTA3</i>	MD10G1051700	1	1075	Nucleus	Chr10
<i>MdCAMTA4</i>	MD13G1121200	1	978	Nucleus	Chr13
<i>MdCAMTA5</i>	MD13G1144300	2	887, 1005	Nucleus	Chr13
<i>MdCAMTA6</i>	MD15G1318400	2	909, 919	Nucleus	Chr15
<i>MdCAMTA7</i>	MD16G1121200	1	978	Nucleus	Chr16
<i>MdCAMTA8</i>	MD16G1157900	2	911, 913	Nucleus	Chr16

Sequence analysis revealed that the apple calmodulin-binding transcription activators are encoded by genes typically containing 12 to 13 exons. The only exceptions are one splice variant of *MdCAMTA5*, which comprises 9 exons, and one isoform of *MdCAMTA6*, which is spanned by 7 exons. Several *MdCAMTA* genes give rise to multiple protein isoforms via alternative splicing. Specifically, *MdCAMTA1* yields three isoforms ranging from 1091 to 1107 amino acids (aa) in length. Conversely, *MdCAMTA2*, *MdCAMTA3*, and *MdCAMTA4* each encode a single isoform, with protein products spanning 1072, 1075, and 978 aa, respectively. Both *MdCAMTA5* and *MdCAMTA6* encode two protein isoforms each, with lengths of 1005 and 887 aa for *MdCAMTA5*, and 1109 and 909 aa for *MdCAMTA6*. Finally, *MdCAMTA7* produces a single protein of 978 aa, while *MdCAMTA8* generates two isoforms of 911 and 913 aa. Taken together, these eight apple genes code for a total of 13 calmodulin-binding transcription activator isoforms.

The theoretical isoelectric points (pI) of the predicted apple calmodulin-binding transcription activators range from 5.28 to 6.89. In silico subcellular localization analysis indicated that all identified proteins are targeted to the nucleus. Furthermore, MEME (Multiple Em for Motif Elicitation) motif analysis (Figure 1) revealed that these apple CAMTAs share a remarkably similar composition of conserved motifs, which exhibit a highly consistent sequential arrangement. The only notable exceptions are the truncated protein isoforms encoded by *MdCAMTA1*, *MdCAMTA5*, and *MdCAMTA6*.

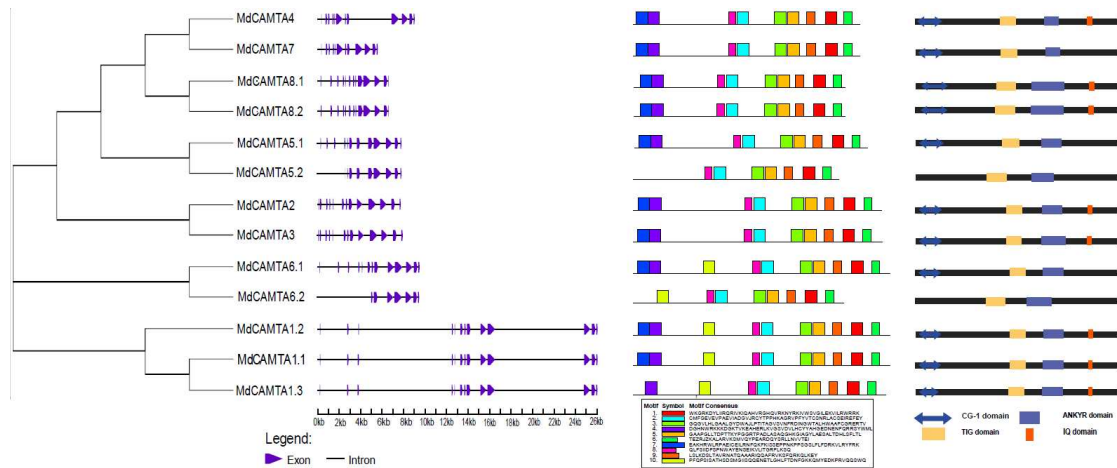


Figure 1. Conserved motifs of the predicted *Malus domestica* CAMTA (MdCAMTA) proteins.

Phylogenetic Relationships of MdCAMTAs and Counterparts from Other Plant Species

To elucidate the evolutionary relationships of the apple calmodulin-binding transcription activators, a comparative phylogenetic analysis was performed using homologous sequences from other dicotyledonous species, specifically *Arabidopsis* (*Arabidopsis thaliana*) and flax (*Linum usitatissimum*). The protein sequences for these species were retrieved from The Arabidopsis Information Resource (TAIR) and FIBexDB databases, respectively. The phylogenetic tree of apple, flax, and *Arabidopsis* CAMTAs—reconstructed via the maximum likelihood method based on multiple sequence alignment executed with the COBALT (Constraint-based Multiple Alignment Tool) algorithm—is presented in Figure 2.

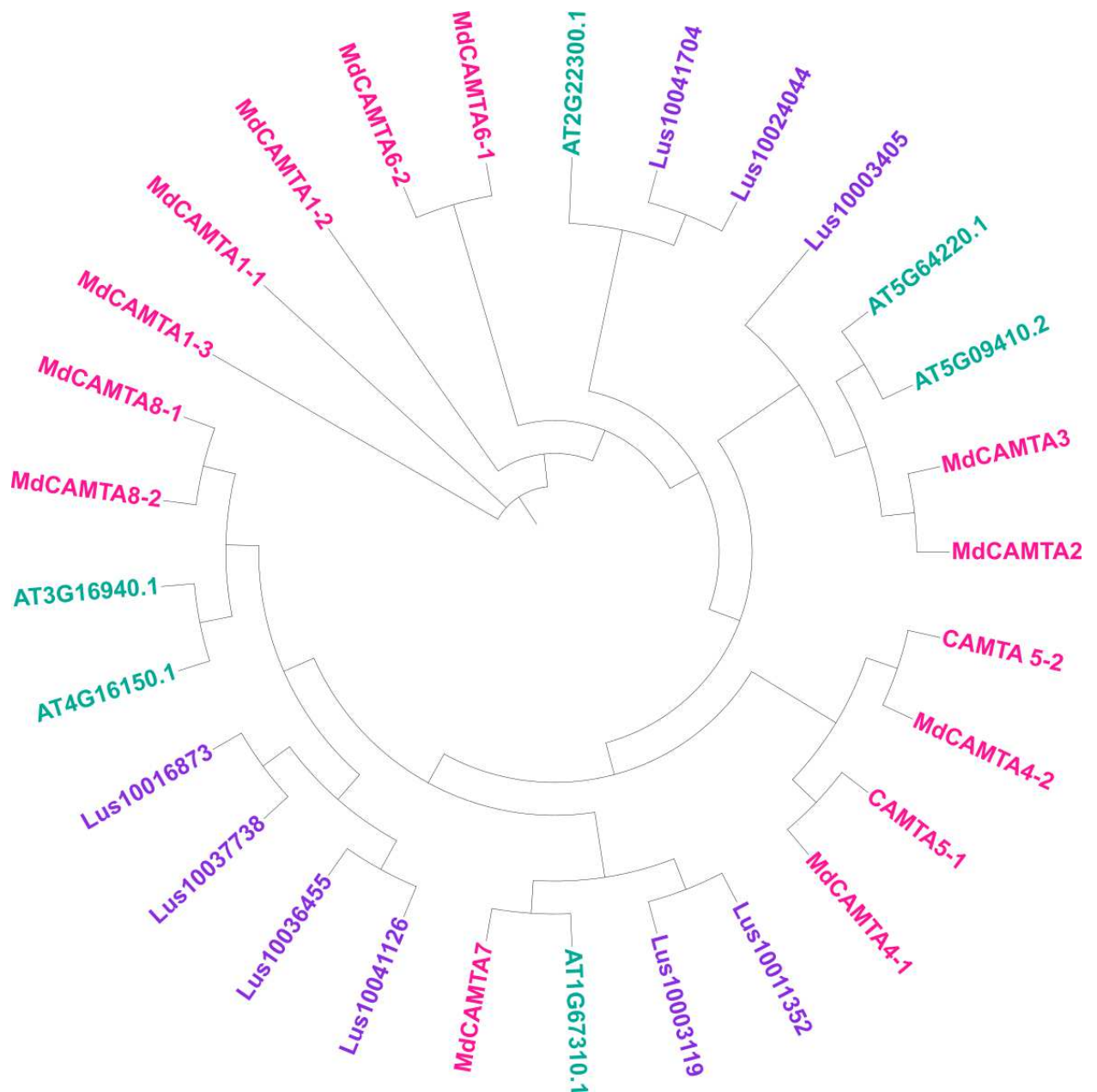


Figure 2. Phylogenetic tree of calmodulin-binding transcription activators from apple (Md), Arabidopsis (At), and flax (Lus) (shown in pink, green, and purple, respectively).

As illustrated, the sequences partition into distinct orthologous groups, each encompassing representatives from all three plant species. Consequently, CAMTA proteins frequently exhibit higher sequence similarity to their interspecific homologs than to paralogs within the same genome, a pattern consistently observed across all analyzed taxa. This topological arrangement provides strong evidence that the divergence of these CAMTA lineages from a common ancestral sequence predated the speciation events that separated the evolutionary ancestors of apple, flax, and Arabidopsis. Concurrently, as anticipated, the alternative protein isoforms encoded by individual apple genes (reaching up to three variants) display a high degree of sequence identity, clustering tightly together.

Analysis of *MdCAMTA* Gene Promoter Regions

Analysis of the promoter regions of apple *CAMTA* genes revealed that they are integral components of a complex regulatory network, interacting with various transcription factors and hormonal inputs that

govern plant growth and development. The critical importance of phytohormone regulation in *MdCAMTA* expression is underscored by the fact that every single identified gene harbored cis-regulatory elements responsive to at least one plant hormone (Figure 3). The lowest diversity of hormonal motifs was observed in the *MdCAMTA3* promoter, which contained elements responsive exclusively to abscisic acid (ABA) and salicylic acid (SA). In contrast, the remaining genes possessed between six and nine distinct types of hormone-related regulatory motifs within their promoter regions. Notably, the regulatory sequences of all *MdCAMTA* genes contained at least one *as-1* and/or TCA-element, indicating that SA is a universal modulator of their transcription. Salicylic acid is a well-established activator of plant defense responses and a key regulator of growth, orchestrating plant immunity, tolerance to both biotic and abiotic stresses, and interactions with the soil microbiome (Koo et al. 2020).

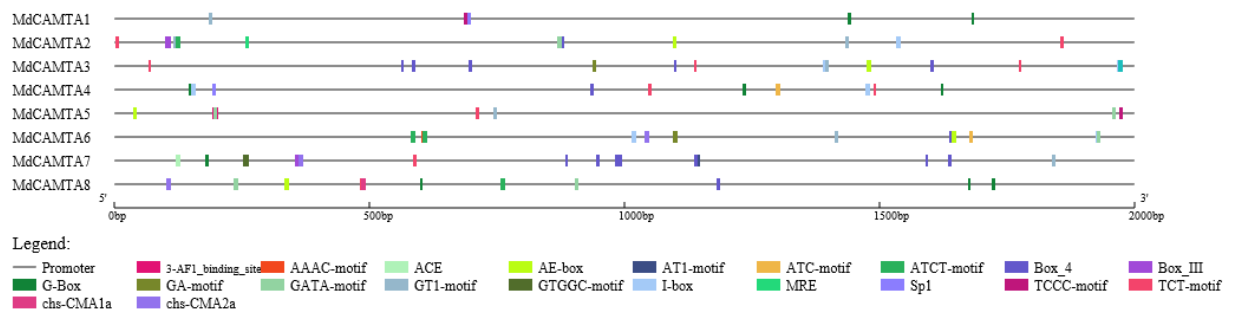


Figure 3. Transcription factor binding sites involved in stress- and hormone-induced signaling pathways identified within the promoter regions of *MdCAMTA* genes.

Methyl jasmonate (MeJA) and its derivatives are implicated in the transcriptional regulation of seven out of the eight *MdCAMTA* genes (87.5%), as evidenced by the presence of CGTCA and TGACG motifs. Methyl jasmonate, jasmonic acid, and its amino acid conjugates—collectively referred to as jasmonates—are essential intracellular regulators that mediate diverse developmental processes, including root growth, pollen production, and plant defense against insects and pathogens (Creelman and Mullet 1997; Kessler and Baldwin 2002). For instance, upon herbivore attack in *Nicotiana attenuata*, jasmonates orchestrate the synthesis of volatile organic compounds that attract natural enemies of the herbivores (Kessler and Baldwin 2001; Mattiacci et al. 1995), as well as the accumulation of secondary metabolites acting as direct defenses, such as the neurotoxin nicotine (Baldwin 1999).

Cis-regulatory elements indicating responsiveness to abscisic acid (ABA) are equally prevalent within the promoter regions of *MdCAMTA* genes. Specifically, seven out of the eight genes (87.5%) harbor at least one ABA-responsive element, such as ABRE, ABRE3a, ABRE4, or the CGTGG motif. Phytohormone ABA modulates plant growth and development through complex transcriptional and post-transcriptional mechanisms, orchestrating processes such as leaf senescence, bud dormancy release, seed germination, and stomatal closure, among others (Chen et al. 2020). Furthermore, ABA triggers a series of signaling cascades essential for establishing tolerance to drought, salinity, and cold stresses (Ingram and Bartels 1996; Bray 1997; Thomashow 1999). However, not all stress-induced plant signaling networks are mediated by ABA. Stress-responsive gene expression profiling in the model plant *Arabidopsis* has demonstrated the existence of at least two ABA-independent signaling pathways, one of which actively mediates responses to drought, salinity, and cold shocks (Tuteja 2007; Ishitani et al. 1997).

Additionally, 75% of *MdCAMTA* genes (six out of eight) are putatively regulated by auxin, as evidenced by the presence of TGA-element or AuxRR-core motifs within their promoter regions. Auxin is a foundational phytohormone that operates both locally and systemically, coordinating the differential growth of various plant organs. Consequently, some researchers have proposed viewing auxin signaling

as a plant analogue to the animal central nervous system—a system absent in plants due to their modular body plan, which requires tolerance to the loss of individual organs and precludes the development of irreplaceable structures (Leyser 2017). Furthermore, the involvement of ethylene in modulating *MdCAMTA* transcription is indicated by the presence of ERE motifs detected in five out of eight genes (62.5%). Ethylene exerts a profound influence on diverse plant developmental programs, including seed germination, fruit ripening, senescence, and abscission, as well as adaptive responses to various soil-borne and environmental stresses, such as flooding, salinity, and soil compaction (Abeles et al. 2012; Mattoo et al. 1991).

Gibberellin-responsive elements, such as GARE-motifs and/or TATC-boxes, were identified in two *MdCAMTA* genes (25%). Gibberellic acid stimulates critical developmental phase transitions throughout the plant life cycle, including seed dormancy release and germination, meristem activation for shoot growth, the juvenile-to-adult vegetative phase transition, and the vegetative-to-reproductive (flowering) transition; it also participates in signaling cascades triggered by fluctuations in light, temperature, and water availability (Gupta and Chakrabarty 2013). Notably, light emerged as a predominant factor influencing *MdCAMTA* transcription, with light-responsive cis-regulatory elements detected within the promoter regions of all family members without exception. The diversity of these motifs varied from a minimum of four types in *MdCAMTA1* to a maximum of 11 types in *MdCAMTA6*. Among these, the most prevalent motifs were the G-box (present in seven out of eight genes, 87.5%), followed by the GT1-motif, TCT-motif, and Box 4 (each identified in 75% of the genes), as well as the GATA-motif and AE-box (62.5%). Additionally, several other light-related elements were sporadically distributed, including the 3-AF1 binding site, AAAC-motif, ACE, ATC-motif, ATCT-motif, AT1-motif, GTGGC-motif, Box III, chs-CMA1a, chs-CMA2a, GA-motif, I-box, LAMP-element, Sp1, and TCCC-motif (Figure 4).

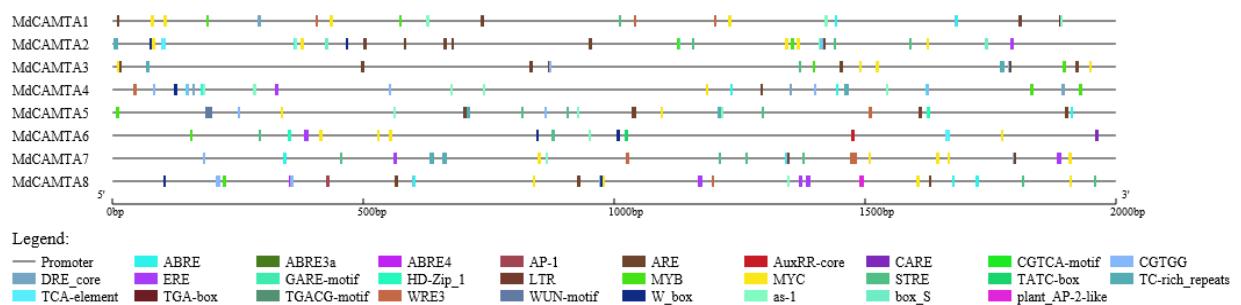


Figure 4. Transcription factor binding sites involved in light-responsive signaling pathways identified within the promoter regions of *MdCAMTA* genes.

Furthermore, *MdCAMTA* genes may play a critical role in mediating stress responses, as evidenced by the presence of stress response elements (STRE), which were identified in 87.5% of the gene promoter regions. An identical proportion of promoters (87.5%) contained anaerobic responsive elements (ARE), which participate in the cellular response to anaerobic or hypoxic stress. Additionally, TC-rich repeats, which are involved in defense and stress responsiveness, were detected in 50% of the analyzed genes. The promoter regions of two genes (25%) harbored dehydration-responsive elements (DRE), which regulate gene expression under drought conditions, while one gene contained a low-temperature responsive element (LTR) involved in cold stress adaptation. Finally, one member of the gene family possessed an AP-1 binding site.

MdCAMTA genes are expected to participate in responses to wounding and pathogen attack. Indeed, WUN-motifs, WRE3 elements, and Box S motifs were identified in 12.5%, 62.5%, and 12.5% of the *MdCAMTA* promoters, respectively. In total, the regulatory regions of six out of eight genes (75%)

harbored at least one of these defense-related elements. Furthermore, the expression of *MdCAMTA* genes may exhibit tissue specificity, as indicated by the presence of the AAGAA-motif, RY-element, GCN4-motif, TCCACGTAGA motif, O2-site, CAT-box, A-box, HD-Zip 1 binding site, and AP2-like binding site among their regulatory sequences. Additionally, the detection of MSA, E2Fb, and MSA-like elements suggests that *MdCAMTA* family members may be involved in cell cycle regulation.

The promoter regions of all *MdCAMTA* genes harbored binding sites for MYB regulatory proteins. These factors are critically involved in mediating biotic and abiotic stress responses, including epigenetic control, phytohormone signaling pathways, cellular differentiation and morphogenesis, and phenylpropanoid biosynthesis. Additionally, each *MdCAMTA* regulatory region contained signature DNA sequences for the binding of MYC family transcription factors. MYC proteins function as key components of jasmonic and salicylic acid signaling networks, coordinating plant growth and developmental processes, responses to diverse environmental stresses, and other biological programs. Furthermore, four out of the eight analyzed promoters (50%) contained the W-box, which serves as the specific DNA binding site for WRKY transcription factors. WRKY factors execute multifaceted functions, including the establishment of disease resistance, stress tolerance, ontogenetic regulations, and hormonal cross-talk.

In conclusion, structural analysis of the genomic regions located immediately upstream of the *MdCAMTA* coding sequences indicates that these genes are integrated into a complex, interconnected network of regulatory processes essential to maintain plant homeostasis and survival. These findings allow us to infer that *MdCAMTA* transcription is dynamically modulated by environmental conditions—such as abiotic and biotic challenges or fluctuations in light intensity—and may exhibit pronounced tissue-specific patterns.

Expression Patterns of *MdCAMTA* Genes

Plant growth, development, and productivity are continuously modulated by diverse environmental and endogenous factors, which trigger dynamic shifts in gene expression as an adaptive strategy. In this study, we evaluated the transcriptional profiles of *MdCAMTA* genes under various biotic and abiotic challenges, alongside their spatial distribution across different apple tissues.

To assess tissue-specific expression patterns, we analyzed the transcription of the *MdCAMTA* gene family across 11 distinct organs and tissues of apple (cv. 'Golden Delicious') using transcriptomic datasets deposited in the NCBI Sequence Read Archive (SRA). The corresponding raw data were retrieved from BioProject PRJNA419119 (Pratas et al. 2018). The results were visualized as a hierarchically clustered heatmap (Figure 5), with row-normalized values ranging from 0.0 (minimum expression level, indicated in purple) to 1.0 (maximum expression level, indicated in pink).

Analysis of the tissue-specific expression of *MdCAMTA* genes revealed that pollen exhibited the most distinct transcriptional profile. While the majority of the family members were transcriptionally silent in this tissue, *MdCAMTA8.2*, *MdCAMTA5.1*, and *MdCAMTA1.3* displayed exceptionally high activity. Notably, *MdCAMTA1.3* exhibited low expression levels across all other analyzed tissues. Similarly, *MdCAMTA5.1* demonstrated a moderate expression level in anthers alongside its strong accumulation in pollen, but remained low in all remaining samples.

Vegetative and floral organs, including sepals, young leaves, stigmas, and petals, exhibited robust expression of the upper gene cluster (*MdCAMTA2*, *MdCAMTA3*, *MdCAMTA7*, *MdCAMTA4*, *MdCAMTA6.1*, and *MdCAMTA8.1*). These family members likely mediate vegetative homeostasis and developmental processes in the green tissues of the plant. Conversely, a sharp decline in the transcriptional activity of *MdCAMTA6.2*, *MdCAMTA1.1*, and *MdCAMTA5.2* was observed in receptacles and styles. This downregulation suggests the operation of alternative regulatory mechanisms in these specific zones, potentially associated with the restriction of certain signaling pathways during fruit set and development.

Taken together, the spatial distribution of *MdCAMTA* transcripts demonstrates pronounced tissue specificity. Notably, pollen possesses a unique expression profile that is virtually reciprocal to that of all

other analyzed tissues. This distinctive pollen signature (characterized by the exclusive activity of *MdCAMTA1.3* and *MdCAMTA5.1* against a background of transcriptional silencing of the remaining family members), coupled with the targeted suppression of specific genes in receptacles and styles, strongly implies that the plant stringently restricts CAMTA-mediated signaling in reproductive organs. This tight spatial compartmentalization likely prevents defense-related physiological trade-offs or autoimmune responses from compromising fertilization and successful reproduction.

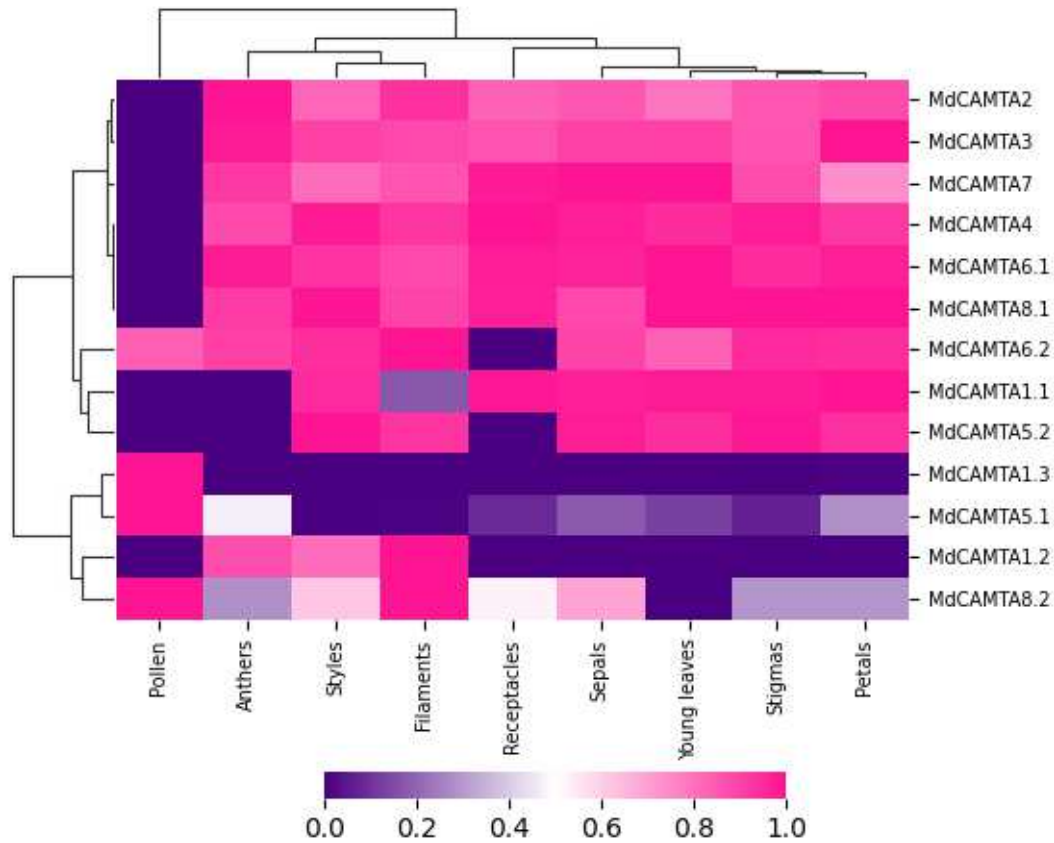


Figure 5. Heatmap of *MdCAMTA* gene expression profiles across different apple tissues. RNA-seq relative expression data from 10 tissues (pollen, anthers, filaments, young leaves, petals, styles, stigmas, sepals, ovaries, and receptacles) were used to reconstruct the spatial expression patterns of *MdCAMTA* genes. The raw data were retrieved from the NCBI Sequence Read Archive (BioProject PRJNA419119). To normalize the dataset, raw TPM counts were $\log_2(X+1)$ transformed prior to clustering. The heatmap colors range from purple (low expression) through white (medium expression) to pink (high expression). The dendrogram on the left represents hierarchical clustering, reflecting the similarity in expression profiles among the identified genes.

To evaluate *MdCAMTA* expression profiles in response to the damaging effects of solar radiation and high temperatures, transcriptomic data from BioProject PRJNA858858 were retrieved from the NCBI Sequence Read Archive (SRA). In this experiment, apple fruits of the 'Honeycrisp' cultivar were subjected to the following treatment conditions: control, sub-sunburn threshold temperature (43–45°C), and sunburn-inducing threshold temperature (47–49°C), with samples collected at 24, 48, and 72 h following the heat stimulus (Waite et al. 2023). As illustrated in the heatmap (Figure 6), the expression profiles of the majority of *MdCAMTA* genes showed no significant deviations from the control group under sunburn-inducing stress. However, prominent transcriptional shifts were observed for the splice variants of *MdCAMTA1.1*, *MdCAMTA1.3*, and *MdCAMTA5.2*, with their response patterns heavily depending on stress intensity. For instance, under sub-sunburn conditions, *MdCAMTA1.3* expression was markedly upregulated (greater than 10-fold) at 48 h post-treatment, before returning to levels near the control values at 72 h. Conversely, under the higher sunburn-inducing temperature, its transcript levels dropped at 24 h, recovered by 48 h, and subsequently exhibited a greater than 10-fold increase relative to

the control at 72 h. In contrast, *MdCAMTA1.1* appeared more sensitive to moderate thermal stress, displaying a progressive downregulation that reached its nadir at 72 h, while remaining relatively unresponsive to critical overheating. The expression profiles of *MdCAMTA5.2* similarly varied as a function of temperature intensity. Taken together, these findings demonstrate that the transcriptional responses of the *MdCAMTA* gene family to solar and thermal injury are highly selective and stress-intensity specific.

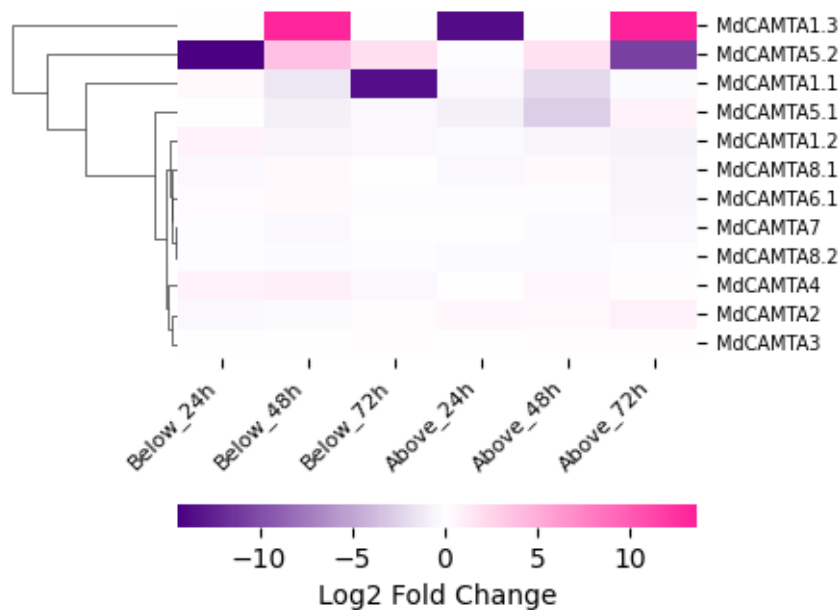


Figure 6. Heatmap of *MdCAMTA* gene expression responses to sunburn conditions. RNA-seq relative expression data from apple peels were used to reconstruct the transcriptional patterns of *MdCAMTA* genes. The raw data were retrieved from the NCBI Sequence Read Archive (BioProject PRJNA858858). To stabilize variance, raw TPM values were $\log_2(X+1)$ transformed prior to clustering. The heatmap colors range from purple (low expression) through white (medium expression) to pink (high expression). The dendrogram on the left represents hierarchical clustering, reflecting the similarity in expression profiles among the identified genes. "Below" and "above" designate samples subjected to sub-sunburn threshold temperatures (43–45°C) and sunburn-inducing threshold temperatures (47–49°C), respectively. The time points 24, 48, and 72 h denote the duration following the heat stimulus.

To evaluate the transcriptional response of *MdCAMTA* genes to hypobaric and hypoxic stress, raw transcriptomic data from Cukrov et al. (2016) were retrieved from the NCBI Sequence Read Archive (SRA) under BioProject PRJNA293208. In this experiment, apple fruits (*Malus domestica*, cv. 'Granny Smith') harvested at commercial maturity were stored at 1°C under normoxic (control) and hypoxic conditions (0.4 kPa and 0.8 kPa oxygen partial pressure), with total RNA extracted after 24 days of hypoxic treatment (Cukrov et al. 2016). To assess *MdCAMTA* transcriptional dynamics, a comparative analysis of differential expression (expressed as $\log_2$ fold change) was performed under both low-oxygen regimes (Figure 7). Profiling of the *MdCAMTA* gene responses to hypoxia induced by altered atmospheric environments revealed that the vast majority of family members exhibited a weak or negligible transcriptional response, clustering tightly within the bottom cluster of the heatmap (Figure 7).

Under more severe hypoxia (0.4 kPa), the expression of *MdCAMTA1.3* was drastically suppressed, dropping to a $\log_2$ fold change ($\log_2FC$) value below -10, whereas *MdCAMTA1.1* and *MdCAMTA4* exhibited marginal transcriptional increases. At 0.8 kPa oxygen, the expression landscape shifted

radically: *MdCAMTA6.2* transcript levels declined significantly, while *MdCAMTA1.2*, conversely, demonstrated a profound upregulation, exceeding a log₂FC of 10. As the sole family member displaying a greater than 1000-fold transcriptional induction, *MdCAMTA1.2* represents a strong candidate for activating hypoxia-adaptation mechanisms in apple. Genes that were sharply downregulated under oxygen-deficient conditions may either participate in silencing energy-intensive processes to conserve cellular resources or serve as diagnostic markers for critical metabolic stress thresholds and regulatory breakdown. The stark contrast between the transcriptional profiles at 0.4 kPa and 0.8 kPa oxygen strongly implies that distinct signaling pathways are sequentially activated upon crossing a specific oxygen concentration threshold.

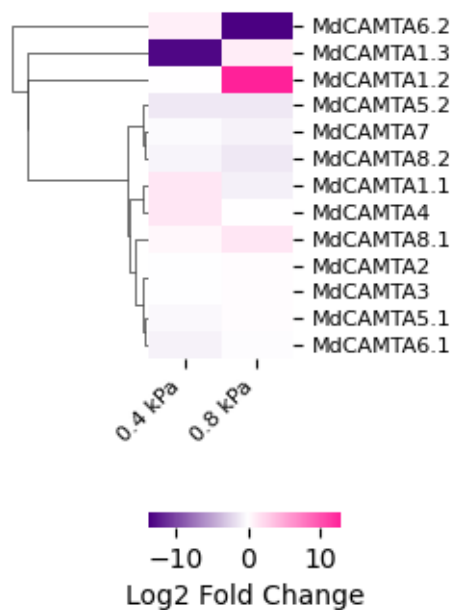


Figure 7. Heatmap of *MdCAMTA* gene expression responses to hypoxic conditions. RNA-seq relative expression data from apple fruit tissues were used to reconstruct the transcriptional patterns of *MdCAMTA* genes. The raw data were retrieved from the NCBI Sequence Read Archive (BioProject PRJNA293208). To stabilize variance, raw TPM values were log₂(X+1) transformed prior to clustering. The heatmap colors range from purple (low expression) through white (medium expression) to pink (high expression). The dendrogram on the left represents hierarchical clustering, reflecting the similarity in expression profiles among the identified genes. The labels "0.4 kPa" and "0.8 kPa" designate samples subjected to different oxygen partial pressure regimes.

To evaluate the transcriptional profiles of *MdCAMTA* genes under nutrient-deficient conditions, raw transcriptomic data from Valentinuzzi et al. (2019) were retrieved from the NCBI Sequence Read Archive (SRA) under BioProject PRJNA505541. This study utilized root samples from apple rootstocks (*Malus × domestica* Borkh., cv. 'M9') subjected to iron deficiency, phosphorus deficiency, or a nutrient-sufficient control regime. The heatmap (Figure 8) illustrates the transcriptional shifts of the *MdCAMTA* gene family in response to both nutritional stresses. The vast majority of the family members (*MdCAMTA4*, *MdCAMTA7*, *MdCAMTA8.1*, *MdCAMTA1.1*, *MdCAMTA5.1*, *MdCAMTA6.1*, *MdCAMTA2*, and *MdCAMTA3*) exhibited no significant alterations in their transcript levels, suggesting they are not actively involved in adapting to these specific mineral scarcities. In contrast, *MdCAMTA1.3* displayed a marginal upregulation under phosphorus deficiency, whereas its activity slightly declined under iron deprivation. *MdCAMTA1.2* demonstrated a slight downregulation (negative log₂ fold change) under both stress

conditions, which was more pronounced during iron deficiency. Notably, *MdCAMTA5.2* exhibited an extraordinary level of induction, with a log₂FC value approaching 10 under both nutrient-deficient regimes. This greater than 1000-fold upregulation positions *MdCAMTA5.2* as a prime candidate for a master regulator of the apple response to mineral nutrient deprivation among the family members, with its transcriptional response being stronger under iron deficiency than under phosphorus deficiency.

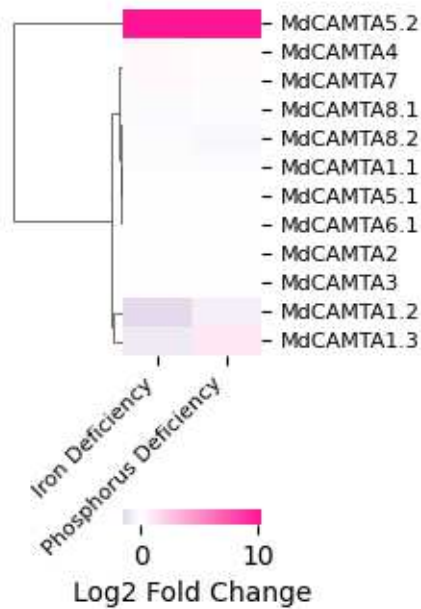


Figure 8. Heatmap of *MdCAMTA* gene expression responses to iron and phosphorus deficiencies. RNA-seq relative expression data from apple roots were used to reconstruct the transcriptional patterns of *MdCAMTA* genes. The raw data were retrieved from the NCBI Sequence Read Archive (BioProject PRJNA505541). To stabilize variance, raw TPM values were log₂(X+1) transformed prior to clustering. The heatmap colors range from purple (low expression) through white (medium expression) to pink (high expression). The dendrogram on the left represents hierarchical clustering, reflecting the similarity in expression profiles among the identified genes.

The impact of biotic stress (pathogen attack) on *MdCAMTA* expression was evaluated using transcriptomic datasets from apple plants infected with either the necrotrophic fungus *Alternaria alternata* apple pathotype or the Apple stem grooving virus (ASGV). Raw RNA-Seq sequencing data from *Malus domestica* 'Starking Delicious' leaves collected at 12, 18, 36, and 72 h post-inoculation were retrieved from the NCBI Sequence Read Archive (SRA) under BioProject PRJNA349086 (Zhu et al. 2017). Additionally, raw data from in vitro-grown ASGV-infected and virus-free plantlets of apple cv. 'Fuji' were obtained from the SRA database under BioProject PRJNA233486 (Chen et al. 2014). Comparative transcriptomic analysis revealed that *MdCAMTA* family members exhibit distinct and differential transcriptional responses to necrotrophic fungal infection by *A. alternata* and systemic viral infection by ASGV.

The transcriptional dynamics of *MdCAMTA* genes in response to the fungal pathogen *Alternaria alternata* are presented in Figure 9. The response of *MdCAMTA* family members is characterized by a selective, wave-like pattern, with the most pronounced expression shifts observed for three specific variants: *MdCAMTA1.3*, *MdCAMTA1.2*, and *MdCAMTA5.2*. Specifically, *MdCAMTA1.3* demonstrated a severe and sustained suppression of expression across all analyzed time points, dropping to a log₂ fold change (log₂FC) value below -10. This greater than 1000-fold downregulation suggests that *MdCAMTA1.3* may act as a negative regulator of the defense response, or alternatively, its transcription is actively suppressed

by fungal virulence factors. In contrast, *MdCAMTA5.2* exhibited a significant transcriptional induction as early as 12 h post-inoculation. Although a transient decline in its activity was observed at 36 h, its transcript abundance rebounded to peak levels by 72 h. This biphasic expression pattern might be linked to recurrent waves of cytosolic calcium influx during the transition from pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) to effector-triggered immunity (ETI) (Ranf et al. 2011). Furthermore, *MdCAMTA1.2* displayed strong activation during the intermediate stage (18 h) before returning to baseline control levels, pointing to its potential involvement in a transient phase of the signaling cascade. The remaining family members, including the *MdCAMTA2*, 3, 4, 7, and 8.1 cluster, remained largely unresponsive to *A. alternata* infection, underscores the narrow functional specialization of individual family members in plant immunity.

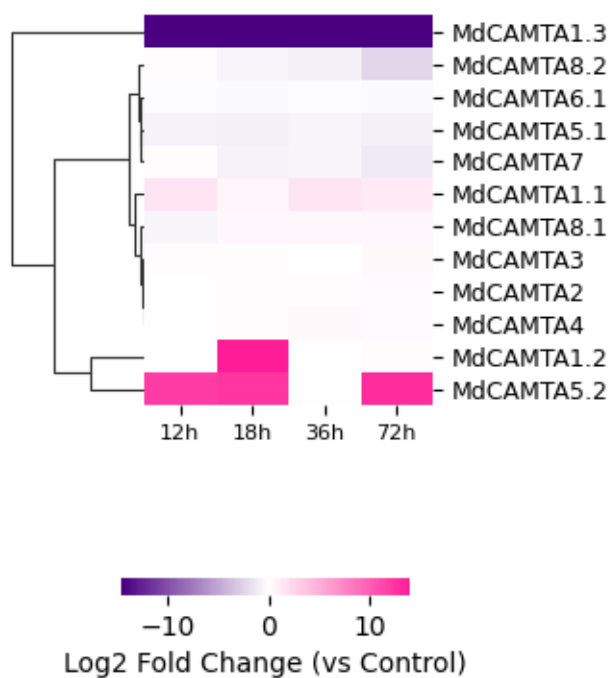


Figure 9. Heatmap of *MdCAMTA* gene expression responses to *Alternaria alternata* infection. RNA-seq relative expression data from apple leaves were used to reconstruct the transcriptional patterns of *MdCAMTA* genes. The raw data were retrieved from the NCBI Sequence Read Archive (BioProject PRJNA349086). To stabilize variance, raw TPM values were $\log_2(X+1)$ transformed prior to clustering. The heatmap colors range from purple (low expression) through white (medium expression) to pink (high expression). The dendrogram on the left represents hierarchical clustering, reflecting the similarity in expression profiles among the identified genes. The labels "12h", "18h", "36h", and "72h" denote the time points post-inoculation with the fungus *Alternaria alternata*.

Upon evaluating the transcriptomic profiles of apple plants infected with ASGV, *MdCAMTA1.2* and *MdCAMTA6.2* emerged as the most virus-sensitive family members (Figure 10). Their expression was drastically suppressed, with $\log_2$ fold change ($\log_2FC$) values dropping to -10 or below, representing a greater than 1000-fold downregulation. In contrast, the remaining *MdCAMTA* genes exhibited substantially more moderate transcriptional responses to the viral infection.

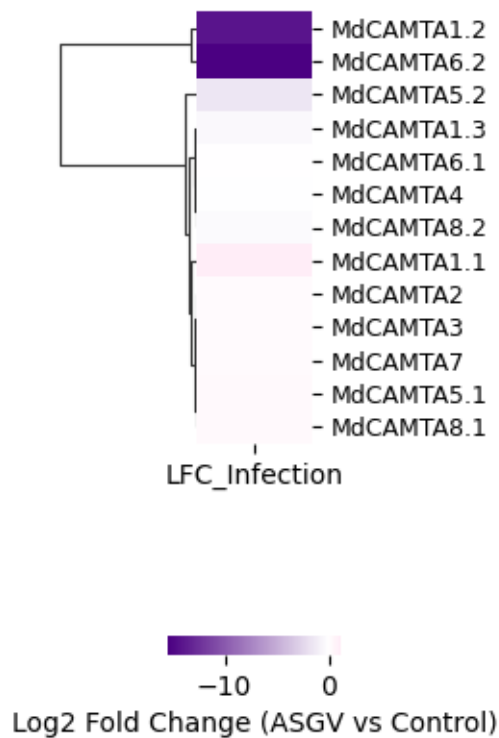


Figure 10. Heatmap of *MdCAMTA* gene expression responses to ASGV infection. RNA-seq relative expression data from apple plantlets were used to reconstruct the transcriptional patterns of *MdCAMTA* genes. The raw data were retrieved from the NCBI Sequence Read Archive (BioProject PRJNA233486). To stabilize variance, raw TPM values were $\log_2(X+1)$ transformed prior to clustering. The heatmap colors range from purple (low expression) through white (medium expression) to pink (high expression). The dendrogram on the left represents hierarchical clustering, reflecting the similarity in expression profiles among the identified genes.

In summary, a clear functional divergence is observable among the family members, dividing them into highly responsive and transcriptomically stable genes. Specifically, *MdCAMTA1.2*, *MdCAMTA1.3*, and *MdCAMTA5.2* dynamically alter their expression profiles in response to diverse environmental cues, including pathogen infections, abiotic stresses, and mineral nutrient deficiencies. Conversely, the *MdCAMTA2*, 3, 4, 7, and 8.1 cluster exhibits pronounced transcriptional inertia under most stress conditions, likely performing housekeeping functions to maintain basal metabolic homeostasis in vegetative tissues.

Conclusion

In this study, eight *MdCAMTA* genes and 13 corresponding alternative splice variants were identified and characterized. Phylogenetic analysis revealed that MdCAMTA proteins partition into two ancient clades that diverged prior to the evolutionary radiation of dicotyledonous lineages, indicating that the structural diversification and evolutionary distances within this family were established during the early stages of higher plant evolution. Promoter architecture and transcriptional profiling demonstrated that *MdCAMTA* activity is tightly linked to light regimes, phytohormone signaling, and the regulatory inputs of MYB, MYC, and WRKY transcription factors. The pronounced tissue-specific expression patterns, particularly the unique transcriptional signature observed in pollen, point to a stringent spatial and temporal control of

CAMTA-mediated signaling by both exogenous and endogenous factors. Furthermore, the highly specific transcriptional responses of these genes to external challenges position *MdCAMTA1.2* and *MdCAMTA5.2* as prime candidates for future functional validation regarding their roles in hypoxia adaptation, systemic viral infection responses, nutritional homeostasis, and antifungal immunity. Overall, these findings establish a comprehensive foundation for detailed functional studies aimed at deciphering the precise molecular mechanisms by which the *MdCAMTA* gene family modulates environmental stress tolerance and resilience in apple.

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Data availability

The datasets analyzed during this study are available in public repositories. Genomic sequences were retrieved from the Genome Database for Rosaceae (GDR) and NCBI GenBank. Transcriptomic data were obtained from the NCBI Sequence Read Archive (SRA) under BioProject accessions PRJNA419119, PRJNA858858, PRJNA293208, PRJNA505541, PRJNA349086, and PRJNA233486. All newly generated analysis results are included within this article.

Consent for Publication

Consent for publication: Not applicable. This study involved exclusively the bioinformatic re-analysis of publicly available genomic and transcriptomic datasets retrieved from the Genome Database for Rosaceae (GDR) and NCBI GenBank/SRA. No live plant materials were collected, cultivated, or sampled by the authors for this study, and no field experiments were conducted. Therefore, specific plant collection guidelines, permissions, or licenses were not required.

Ethics Approval and Consent to Participate

Ethics approval and consent to participate: Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

P. K. and O. U. conceived and designed the study. A. M. performed the Multiple Em for Motif Elicitation (MEME) analysis and prepared Figure 1. P. K. performed the remaining bioinformatic analyses, prepared the figures, and wrote the main manuscript text. All authors reviewed and approved the final manuscript.

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