

Article

Genome-Wide Identification of the *CaMED* Gene Family in Pepper (*Capsicum annuum* L.) and Functional Characterization of *CaMED25a* in the Heat Stress Response

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Abstract

Pepper (*Capsicum annuum* L.) is an important horticultural crop whose growth, development, and yield formation are severely constrained by heat stress. The Mediator complex is a key transcriptional co-regulator in plants and plays important roles in developmental processes and stress responses. However, the MED gene family and its functions in heat stress responses remain largely unexplored in pepper. Using the chromosome-level reference genome of the cultivated pepper (*Capsicum annuum* var. *annuum*) cultivar Zhangshugang, a total of 49 *CaMED* genes were identified and classified into four conserved Mediator modules, namely the head, middle, tail, and kinase modules. Comprehensive bioinformatic analyses showed that *CaMED* genes are evolutionarily conserved across species, whereas differences in gene structure and sequence characteristics among family members may contribute to their functional diversification. Promoter analysis further showed that these genes contain abundant *cis*-acting elements related to light, phytohormone, and stress responses. Transcriptome analysis of the 49 identified *CaMED* genes showed distinct tissue-specific expression patterns, with many members showing preferential expression during early flower development and late placenta development. Furthermore, expression profiling of all *CaMED* genes using publicly available transcriptome datasets under 42 °C heat-stress conditions, followed by RT-qPCR validation of selected candidates, showed that *CaMED25a* displayed a relatively stable heat-responsive expression pattern. Virus-induced gene silencing of *CaMED25a* compromised heat tolerance in pepper plants under heat stress, as evidenced by increased H₂O₂ accumulation and significantly reduced expression of heat defense-related genes, including *CaHSP18*, *CaHSP25.9*, and *CaHSP70.1*. Taken together, this study provides an integrated analysis of the pepper *CaMED* gene family and reveals the positive contribution of *CaMED25a* to heat stress tolerance. These findings lay the groundwork for subsequent studies on *CaMED* gene function and the molecular regulation of high-temperature responses in pepper.



Academic Editor: Haijun Gong

Received: 7 May 2026

Revised: 6 June 2026

Accepted: 22 June 2026

Published: 24 June 2026

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Keywords: *Capsicum annuum* L.; Mediator complex subunit; gene expression; heat stress; *CaMED25a*

1. Introduction

The Mediator complex is a key coactivator in eukaryotic transcriptional regulation and is involved in nearly all transcriptional regulatory activities in eukaryotes [1]. As a molecular bridge between gene-specific transcription factors and the Pol II transcriptional machinery, the Mediator complex plays central roles in promoting transcription pre-initiation complex assembly, mediating transcriptional reprogramming, and integrating upstream regulatory signals [2,3]. The canonical Mediator complex is composed of multiple functionally specialized subunits and is generally organized into four modules: Head, Middle, Tail, and the dissociable Kinase/CDK module [4]. These modules perform distinct functions in transcriptional regulation [5,6]. In plants, increasing evidence indicates that MED subunits are involved not only in growth and development, hormone signaling, and immune regulation, but also in abiotic stress responses [7–10]. For example, MED25 regulates drought and salt stress responses [11], MED2 positively regulates cold stress responses [12], and MED8 participates in oxidative stress responses [13].

Heat stress is one of the major abiotic stresses that restrict plant growth, development, and yield formation [14]. Under heat stress, plants activate complex defense mechanisms to perceive heat signals and initiate heat stress responses. These mechanisms include heat shock transcription factor-mediated synthesis and accumulation of heat shock proteins, activation of antioxidant enzyme systems to maintain reactive oxygen species homeostasis, and epigenetic regulation of heat-responsive gene expression, thereby alleviating heat-induced damage [15–17]. Previous studies have shown that the Mediator complex is also involved in transcriptional regulation associated with plant heat responses. For instance, MED17 integrates jasmonic acid and auxin signaling to regulate thermomorphogenesis [18], MED14 participates in the activation of heat-responsive genes [19], and CDK8 and MED12 are associated with the establishment and maintenance of heat stress memory [20]. These findings highlight the importance of the Mediator complex in heat-stress adaptation. However, studies on the role of the MED family in heat responses have mainly focused on model plants such as *Arabidopsis thaliana*, whereas its functions in horticultural crops remain poorly understood.

Pepper (*Capsicum annuum* L.) is widely cultivated as both a vegetable and spice crop and has considerable economic importance in global horticulture. Heat stress represents a major environmental constraint on pepper growth and development. Under high temperature conditions, particularly during summer production, temperatures above 32 °C can increase flower abscission, reduce fruit set, cause leaf injury, suppress photosynthesis, and impair reproductive growth, ultimately affecting yield formation [21,22]. Plant adaptation to heat stress largely depends on the transcriptional regulation of heat response genes, and Mediator subunits play critical roles in integrating heat signals and regulating the expression of heat response genes. Although MED gene families have been identified in several plant species [23–25], the MED gene family in pepper has not yet been systematically characterized, and its response mechanism under heat stress remains unclear. Therefore, genome-wide identification of pepper MED genes, combined with heat-stress expression profiling to screen key candidate members, is necessary for clarifying the possible functions of the CaMED family in pepper development and heat stress adaptation.

Considering the regulatory significance of the Mediator complex in plant development and stress responses, together with the detrimental effects of heat stress on pepper production, this study conducted a genome-wide identification of CaMED family members in pepper. Their physicochemical features, phylogenetic relationships, syntenic associations, promoter cis-acting elements, and tissue expression patterns were analyzed in detail. In addition, expression profiling under 42 °C heat treatment and qRT-PCR validation indicated that most CaMED genes were responsive to heat stress, suggesting that this family

may be involved in pepper heat-stress regulation. Based on these analyses, *CaMED25a* was selected as a candidate gene associated with heat stress responses. Furthermore, the potential involvement of *CaMED25a* in pepper heat-stress responses was preliminarily assessed using virus-induced gene silencing (VIGS). Overall, this study offers a basis for future functional analyses of the pepper *MED* gene family and provides useful insights for screening heat-responsive genes and improving thermotolerance in pepper breeding.

2. Materials and Methods

2.1. Plant Materials and Cultivation Conditions

The cultivated pepper (*Capsicum annuum* L.) cultivar ‘Zhangshugang’ and *Nicotiana benthamiana* were selected as the plant materials for this study. The pepper seeds were supplied by the Pepper Research Team, Hunan Agricultural University. Plants were grown in a controlled-environment growth room set to a 28 °C/20 °C day/night temperature regime, with a 16 h light/8 h night cycle, approximately 10,000 lx light intensity, and 60–70% relative humidity. *N. benthamiana* was used in subsequent transient expression experiments.

2.2. Identification of *CaMED* Gene Family Members

Arabidopsis *MED* protein sequences were retrieved from the TAIR database and used as queries to identify *MED* family members in pepper. The genome assembly and annotation files of the cultivated pepper (*Capsicum annuum* var. *annuum*) cultivar Zhangshugang were downloaded from the Pepper Genome Database (<https://ted.bti.cornell.edu/ftp/pepper/genome/Zhangshugang/>, accessed on 4 June 2025) [26]. Candidate *CaMED* proteins were identified by BLASTP searches against the Zhangshugang protein database with an E-value threshold of 1×10^{-5} . After redundant sequences were removed, *MED* related conserved domains from the Pfam database were used for further screening with the *hmmsearch* program in HMMER (v3.3.2) [27]. The domain composition of each candidate was then confirmed using PROSITE (<https://prosite.expasy.org/> (accessed on 4 June 2025)) and SMART (<http://smart.embl.de/>, accessed on 4 June 2025) [28,29], and the final members of the pepper *CaMED* gene family were identified.

Protein characteristics of the identified *CaMED* members, including sequence length, molecular weight, and theoretical isoelectric point, were calculated with the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>, accessed on 5 June 2025) [30]. Subcellular localization was predicted using Plant mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>, accessed on 5 June 2025) [31]. *CaMED* proteins were assigned to the Head, Middle, Tail, and Kinase modules based on the established classification of the Arabidopsis *MED* family [23].

2.3. Evolutionary Relationship Analysis of the *CaMED* Family

MED protein sequences from pepper, Arabidopsis, and tomato were aligned with MEGA 11. Based on the alignment results, a Neighbor-Joining phylogeny was inferred under the JTT + G protein substitution model, and node reliability was assessed using 1000 bootstrap replicates [32]. The constructed phylogenetic tree was then displayed and annotated with the iTOL online tool (<https://itol.embl.de/>, accessed on 7 June 2025) [33].

2.4. Prediction of *Cis*-Regulatory Elements in *CaMED* Promoters

For each *CaMED* gene, the 2-kb genomic region located before the translation initiation codon was retrieved and analyzed with PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/> (accessed on 8 June 2025)) to identify potential *cis*-acting elements. The predicted motifs were grouped according to their putative regulatory roles, and the summarized data were plotted using the Python (v3.10.2) matplotlib package [34].

2.5. Chromosomal Localization, Gene Structure and Synteny Analysis

Gene annotation files of *CaMED* family members were obtained from the Zhangshugang pepper genome. Exon–intron structures were visualized using the GSDS online tool (<https://gsds.gao-lab.org/index.php>, accessed on 10 June 2025) [35], whereas genomic positions of *CaMED* genes on chromosomes were mapped using TBtools-II (v2.454) [36].

BLASTP analysis was performed using *CaMED* protein sequences ($E\text{-value} \leq 1 \times 10^{-10}$). Intraspecific syntenic relationships among *CaMED* genes were identified using MCScanX (v1.0.0), and the corresponding gene duplication types were classified using duplicate_gene_classifier (v1.0.0) [37]. For comparative collinearity analysis, Arabidopsis and tomato genome assemblies together with their annotation files were downloaded from Phytozome v13 and the Sol Genomics Network, respectively. MCScanX was further used to analyze interspecific syntenic relationships between pepper and Arabidopsis, as well as between pepper and tomato. The final collinearity results were visualized using TBtools-II (v2.454).

2.6. Transcriptome-Based Analysis of Tissue-Specific and Heat-Responsive Expression Patterns of *CaMED* Genes

Public RNA-seq datasets deposited in PepperHub (<http://lifenglab.hzau.edu.cn/PepperHub/index.php>, accessed on 10 July 2025), an informatics hub for pepper genomic and transcriptomic resources [38], were used to evaluate *CaMED* transcript abundance in different pepper tissues and under high-temperature treatment. The dataset included samples from 48 tissues, as well as leaf and root samples collected at 1, 1.5, 3, 6, 12, and 24 h following 42 °C heat-stress treatment, with the 0 h samples used as controls. Gene expression values were represented as TPM (Transcripts Per Million) according to the original transcriptome analysis pipeline described by Tang et al. [39]. Heatmaps were generated using TBtools-II (v2.454). Gene expression data were Z-score normalized per row and hierarchically clustered (Euclidean distance, average linkage) to compare transcriptional profiles.

2.7. Subcellular Localization

The complete coding region of *CaMED25a*, excluding the stop codon, was amplified from cDNA of the pepper cultivar ‘Zhangshugang’ using 2 × Phanta Max Master Mix (Dye Plus) (Vazyme, Nanjing, China). The amplified fragment was inserted into the pCambia1300-35S-GFP vector using the Uniclone One Step Seamless Cloning Kit (Genesand, Beijing, China), generating the 35S: *CaMED25a*-GFP fusion construct. After sequencing verification, the recombinant vector was introduced into *Agrobacterium tumefaciens* strain GV3101 competent cells (WeidiBio, Shanghai, China), with 35S: GFP serving as the control. According to the *Agrobacterium*-mediated transient expression method in tobacco [40], bacterial suspensions harboring 35S: *CaMED25a*-GFP or 35S: GFP were separately mixed with the nuclear localization marker suspension and infiltrated into leaves of *Nicotiana benthamiana*. GFP fluorescence and nuclear marker signals were observed at 48 h post-infiltration using a confocal laser scanning microscope (Leica DMI8, Wetzlar, Germany). The subcellular localization of *CaMED25a* was determined according to the fluorescence distribution. Primer sequences are listed in Table S1.

2.8. Heat Stress Treatment and RT-qPCR Analysis

The publicly available heat-stress transcriptome datasets used in this study were generated from the pepper line 6421, whereas RT-qPCR validation was performed using the cultivar Zhangshugang, which served as the reference genome for *CaMED* gene identification. Pepper plants selected for heat treatment were initially grown under standard conditions in a controlled-environment growth room until reaching the required develop-

mental stage. The plants were then transferred to a constant temperature and humidity chamber (Hanyu Instrument HWS-50, Shanghai, China) and subjected to heat treatment at 42 °C for the indicated time periods. Leaf samples were harvested at 0, 1, 2, 3, 4, 5, and 6 h after heat exposure, with three biological replicates prepared for each sampling point. All harvested samples were rapidly frozen in liquid nitrogen and kept at −80 °C before RNA extraction.

Total RNA was isolated from pepper leaves using the Eastep® Super Total RNA Extraction Kit (Promega, Shanghai, China) according to the manufacturer's instructions. Qualified RNA was subsequently reverse-transcribed using the HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). *CaUBI* served as the internal control gene. For each sample, For each treatment, three independent biological replicates were collected, and each biological replicate consisted of pooled samples from five individual pepper plants. Relative transcript levels were determined using the $2^{-\Delta\Delta C_t}$ method. Statistical analyses and data visualization were performed using GraphPad Prism (v10.5.0). Primer information is provided in Table S1.

2.9. VIGS Validation of *CaMED25a* and Heat Stress Treatment

A 299-bp fragment (886–1184 bp of the *CaMED25a* coding sequence) was designed using the VIGS Tool (<https://vigs.solgenomics.net/>, accessed on 28 July 2025) in the Sol Genomics Network and cloned into the *Sma*I restriction enzyme-linearized (Thermo Fisher Scientific, Waltham, MA, USA) pTRV2-C2b vector by homologous recombination using the Uniclone One Step Seamless Cloning Kit (Genesand, Beijing, China). The resulting construct was designated TRV2:*CaMED25a*. TRV2:00 and TRV2:*PDS* were used as the negative and positive controls, respectively.

The TRV-mediated VIGS assay was performed following the pepper infiltration protocol reported by Zhou et al. [41]. Approximately 4 weeks after infiltration, when TRV2:*PDS* plants showed a photobleaching phenotype, the silencing efficiency in TRV2:*CaMED25a* plants was evaluated using RT-qPCR. Silenced and control plants at the same developmental stage were transferred to a constant temperature and humidity chamber and exposed to 42 °C for 8–12 h until clear phenotypic differences were observed. Leaf samples were collected before and after heat treatment for DAB staining and RT-qPCR analysis of related genes. For each treatment and time point, three independent biological replicates were sampled and analyzed. Primer information is provided in Table S1.

2.10. DAB Staining

To detect H₂O₂ accumulation in leaves under heat stress, histochemical analysis was conducted using a freshly prepared DAB (3,3'-diaminobenzidine) solution (1 mg/mL, pH 3.8) prepared from DAB tetrahydrochloride hydrate powder (Coolaber, Beijing, China). Leaf samples were incubated in freshly prepared DAB solution (1 mg/mL, pH 3.8) under dark conditions for 6–8 h. After staining, chlorophyll was removed by heating the leaves in 95% ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) until the tissues became sufficiently decolorized. The decolorized leaves were then transferred to a preservation solution consisting of glycerol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and 1 × PBS buffer (Coolaber, Beijing, China) mixed at a 1:1 (v/v) ratio, and representative phenotypes were photographed using a digital camera [42]. The intensity of brown precipitate accumulation was used to indicate the relative level of H₂O₂ accumulation.

3. Results

3.1. Identification, Structural Features, and Physicochemical Properties of CaMED Family Members

To gain insights into the composition, evolutionary conservation, and potential functional diversification of the *CaMED* gene family in pepper, *CaMED* family members were identified from the Zhangshugang pepper genome and subjected to comprehensive analyses of their structural characteristics and physicochemical properties. Through BLASTP homology searches complemented by conserved domain validation against the CDD, a total of 49 *CaMED* genes were mined from the Zhangshugang pepper genome (Table S2). Chromosomal mapping showed that these *CaMED* genes were not evenly located among the 12 pepper chromosomes. Chromosome 4 harbored the highest number of *CaMED* genes, with 13 members, whereas only one member was detected on chromosome 10 (Figure 1). Physicochemical analysis revealed clear variation in the predicted *CaMED* proteins, including amino acid length (79–2267 aa), molecular weight (9.4–250.97 kDa), and theoretical isoelectric point (4.36–11.89). Based on the modular classification of the Arabidopsis Mediator complex, the 49 identified *CaMED* family members were assigned to four modules, including 18 members in the Head module, 10 in the Middle module, 15 in the Tail module, and 6 in the Kinase module (Table S2). Gene structure analysis revealed marked structural differences among *CaMED* genes (Figure S1). Among them, *CaMED13a* contained the largest number of exons, whereas *CaMED4a*, *CaMED8a*, *CaMED17b*, and *CaMED32a* each contained only one exon, suggesting that the *CaMED* gene family has undergone considerable structural divergence during evolution.

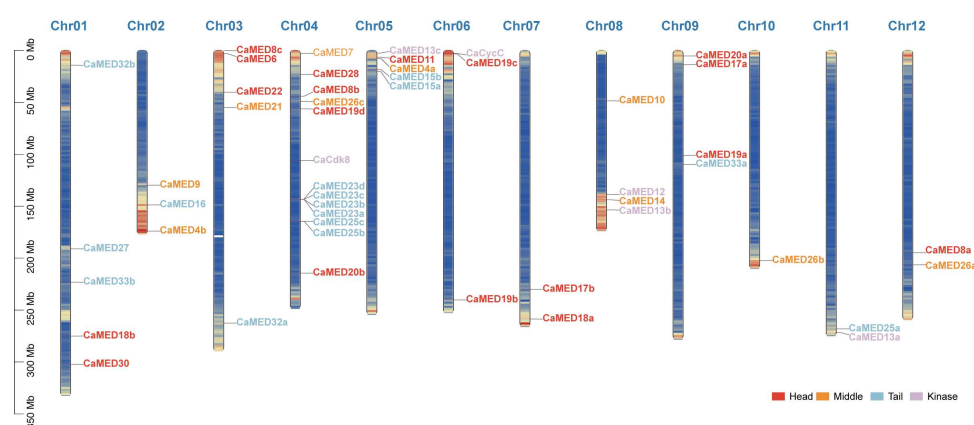


Figure 1. Chromosomal distribution of *CaMED* genes in pepper. Different colors represent members belonging to different Mediator modules.

3.2. Evolutionary Relationship Analysis of the *CaMED* Gene Family

To further elucidate the evolutionary relationships and evolutionary conservation between pepper MED proteins and those from other plant species, MED protein sequences from pepper, Arabidopsis, and tomato were aligned using MEGA, and an NJ phylogenetic tree was then generated (Figure 2). The results showed that most MED proteins from pepper, Arabidopsis, and tomato clustered with their corresponding homologs in the phylogenetic tree, indicating close evolutionary relationships. For example, *CaMED21*, *SIMED21*, and *AtMED21*; *CaMED25a*, *CaMED25b*, *SIMED25b*, and *AtMED25*; as well as *CaMED9*, *SIMED9*, and *AtMED9* were grouped into the same respective clades. These results suggest that MED proteins are highly homologous and evolutionarily conserved across different species. However, not all members within the same module clustered closely together. For instance, the Head module members *CaMED8a*, *CaMED8b*, *CaMED8c*, *SIMED8*, and *AtMED8* were relatively distant from one another in the phylogenetic tree. These findings indicate that although the *CaMED* family is generally conserved during

long-term evolution, some members may have undergone sequence divergence to varying degrees, which may provide a basis for functional diversification.

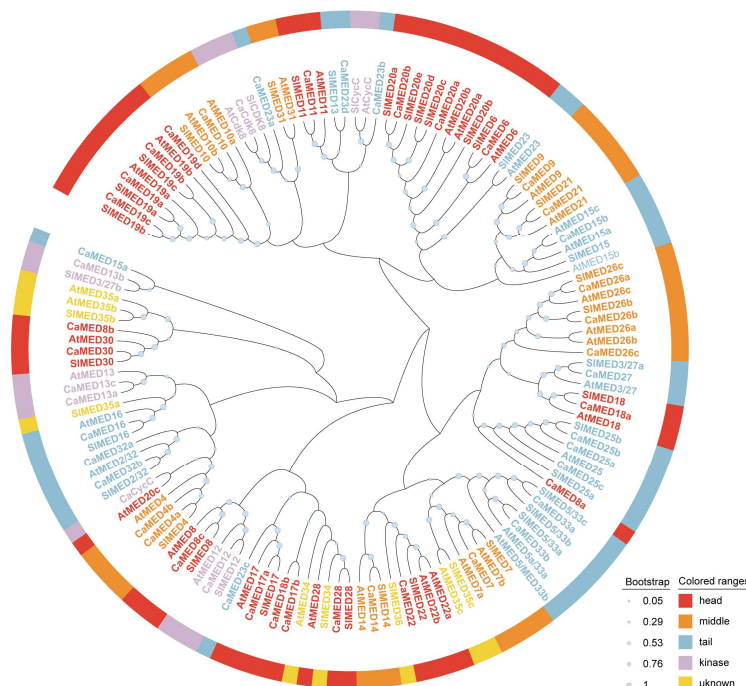


Figure 2. Phylogenetic analysis of CaMED proteins in different plant species. The tree was inferred using the neighbor-joining approach and illustrates the clustering patterns of MED proteins from pepper (*Capsicum annuum*, Ca), Arabidopsis (*Arabidopsis thaliana*, At) and tomato (*Solanum lycopersicum*, Sl). Different colors represent members belonging to different Mediator modules.

3.3. Intraspecific and Interspecific Synteny Analysis of the CaMED Gene Family

To clarify the expansion characteristics and evolutionary conservation of the pepper CaMED family, synteny analyses were conducted within pepper and among different species. In the pepper genome, five pairs of syntenic CaMED genes were identified (Figure 3A), including CaMED8a/CaMED8b, CaMED26a/CaMED26c, CaMED15a/CaMED15b, CaMED19b/CaMED19c, and CaMED19a/CaMED19c. Gene duplication classification showed that dispersed duplication and singleton genes were the predominant duplication types among CaMED family members (Table S3), suggesting that gene duplication events contributed to the retention and evolution of some CaMED genes. Interspecific synteny analysis identified 35 homologous syntenic gene pairs between pepper and tomato, whereas only 6 such pairs were detected between pepper and Arabidopsis (Figure 3B). These results suggest stronger conservation of the CaMED family between closely related Solanaceae species.

3.4. Analysis of Cis-Acting Elements in CaMED Gene Promoters

To investigate the potential transcriptional regulatory mechanisms of CaMED genes and assess their possible roles in hormone signaling and abiotic stress responses, *cis*-acting elements in their promoter regions were analyzed. *Cis*-acting elements were predicted within the 2-kb upstream promoter regions of the 49 CaMED genes. The analysis revealed that the promoters of CaMED family members contained abundant elements related to light response, phytohormone signaling, and stress regulation (Figure 4). Among these elements, light-responsive motifs showed the broadest distribution and were detected in all CaMED promoters. The hormone-related elements were mainly associated with abscisic acid, methyl jasmonate, auxin, salicylic acid, and gibberellin responses. Stress-associated

motifs primarily included elements related to low-temperature response, drought response, and defense/stress regulation.

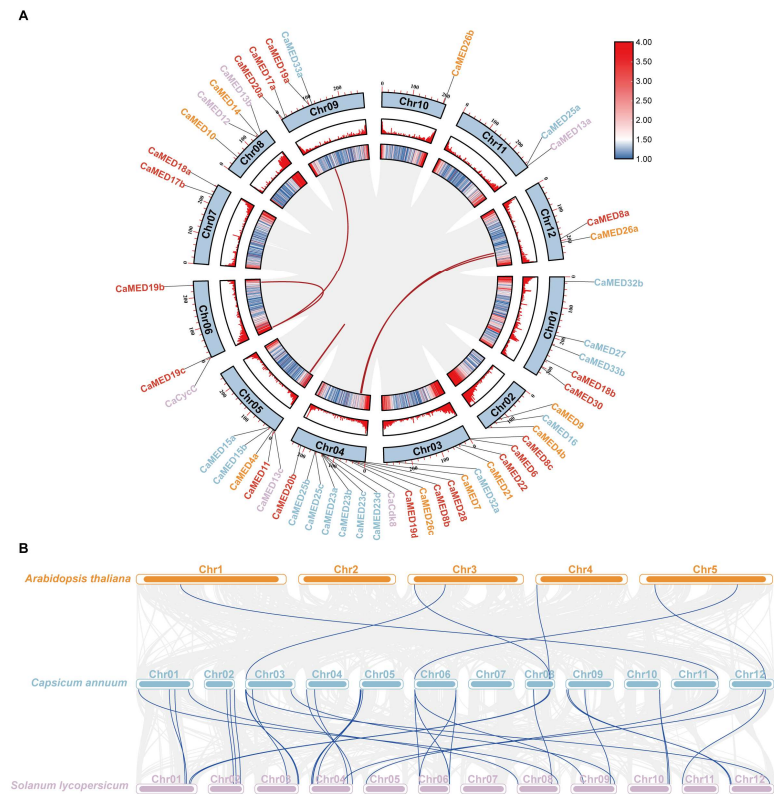


Figure 3. Intrasppecific and interspecific synteny analysis of *CaMED* genes. **(A)** Genomic collinearity of *CaMED* genes within the pepper genome. Red lines indicate collinear gene pairs generated by genomic duplication events. **(B)** Comparative synteny of *MED* genes among *Arabidopsis*, tomato, and pepper. Blue lines connect orthologous gene pairs across different species.

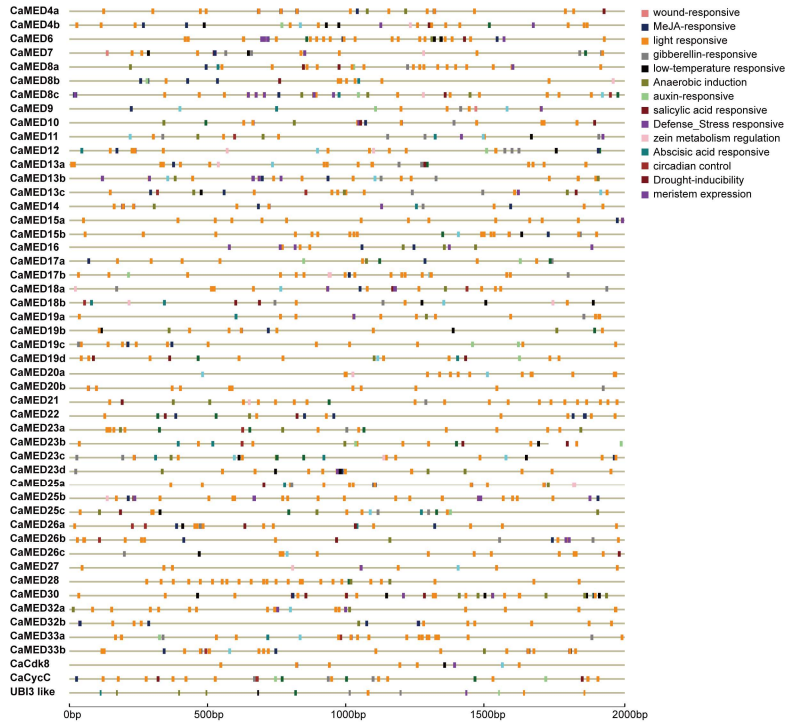


Figure 4. Distribution of *cis*-acting elements in *CaMED* gene promoters.

Overall, diverse *cis*-acting elements were distributed throughout the promoter regions of *CaMED* genes, but their number and composition varied considerably among different members. For example, the *CaMED25a* promoter contained 13 light-responsive elements, 4 methyl jasmonate-responsive elements, 4 abscisic acid-responsive elements, 1 gibberellin-responsive element, and 1 drought-responsive element. It also contained anaerobic induction and circadian rhythm-related elements. These *cis*-acting elements suggest potential roles of *CaMED25a* in responses to light, phytohormone signaling, and abiotic stresses; however, their biological functions require further experimental validation. In contrast, the *CaMED25b* promoter contained 22 light-responsive elements, 6 abscisic acid-responsive elements, and 6 methyl jasmonate-responsive elements, whereas the *CaMED25c* promoter lacked methyl jasmonate- and abscisic acid-responsive elements.

3.5. Transcriptome-Based Tissue-Specific Expression Patterns of *CaMED* Genes

To investigate the tissue-specific expression patterns of *CaMED* genes, publicly available transcriptome datasets were retrieved from the PepperHub database and analyzed across 48 tissues, including roots, stems, leaves, flowers, ovaries, petals, anthers, fruits, pericarps, seeds, and placentas at different developmental stages (Figure 5). The results showed that *CaMED* genes exhibited substantial differences in expression levels among tissues, indicating strong spatiotemporal expression specificity. Overall, most *CaMED* members showed relatively high expression during early flower development (F1-F2) and late placenta development (T7-T11). In leaves, only *CaMED33a* and *CaMED8a* showed relatively high expression levels, whereas most other members were expressed at low levels, suggesting that this gene family may be closely associated with the growth and development of reproductive organs in pepper. In addition, several members showed clear tissue-preferential expression. For instance, *CaMED32a* was highly expressed in seeds, *CaMED25a* maintained high expression throughout early flower development (F1-F5), whereas *CaMED25c* showed low expression levels in most tissues. These results indicate that *CaMED* gene family members exhibit marked expression divergence among different tissues, suggesting that they may perform diverse biological functions during pepper growth and development.

3.6. Transcriptome-Based Analysis of Heat-Responsive Expression Patterns of *CaMED* Genes

To assess the heat responsiveness of the *CaMED* gene family, publicly available heat-stress transcriptome datasets retrieved from the PepperHub database were analyzed. The datasets included pepper leaves and roots sampled at 1, 1.5, 3, 6, 12, and 24 h after heat treatment (Figure 6). The results showed that *CaMED* gene family members exhibited distinct tissue-specific responses to heat stress. In leaves, most *CaMED* genes showed increased expression after heat treatment, except for *CaMED13a* and *CaMED32a*, which exhibited only minor changes in expression. In roots, however, different members displayed more variable response patterns, with both upregulated and downregulated expression observed.

Notably, *CaMED4a*, *CaMED18a*, *CaMED25a*, *CaMED19c*, *CaMED19b*, *CaMED4b*, *CaMED12*, *CaMED33a*, *CaMED14*, *CaMED13c*, *CaMED16*, *CaMED21*, *CaCycC*, *CaMED15a*, *CaMED17a*, *CaMED19a*, *CaMED30*, and *CaMED18b* showed increased expression in both leaves and roots following heat treatment. These results indicate that *CaMED* genes are generally responsive to heat stress, although their response patterns differ among family members. The heat-induced expression of several *CaMED* genes in both above-ground and belowground tissues further indicates their potential involvement in pepper heat-stress responses.

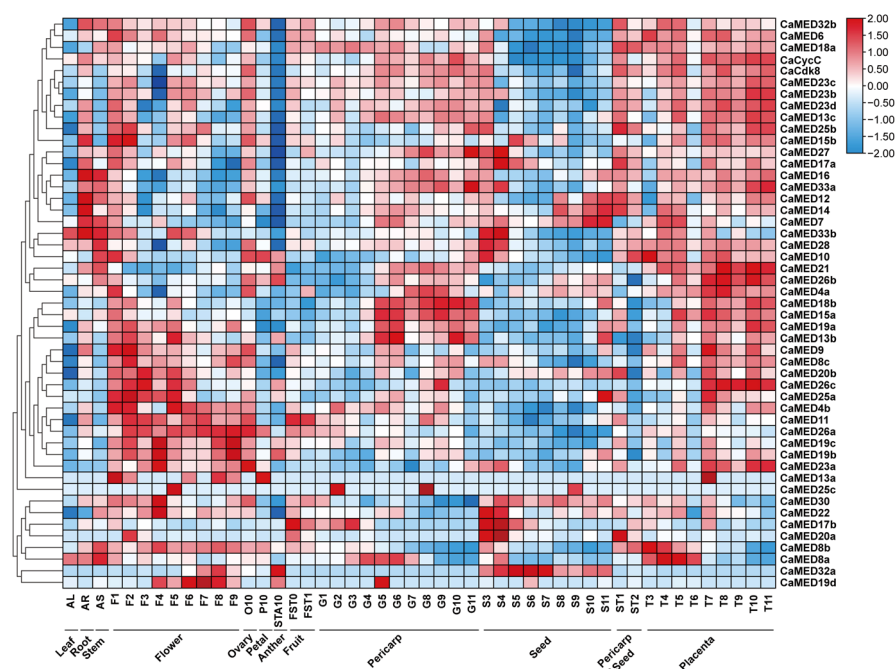


Figure 5. Expression patterns of pepper *CaMED* genes in different tissues and organs. The heatmap was generated using PepperHub RNA-seq datasets to illustrate the transcript abundance of *CaMED* family members across different tissues and organs. The analyzed tissues and organs included mature leaves (AL), developing roots (AR), developing stems (AS), flowers (F1–F9), ovaries (O10), petals (P10), anthers (STA10), fruits (FST0–FST1), pericarps (G1–G11), seeds (S3–S11), pericarps and seeds (ST1–ST2), and placentas (T3–T11).

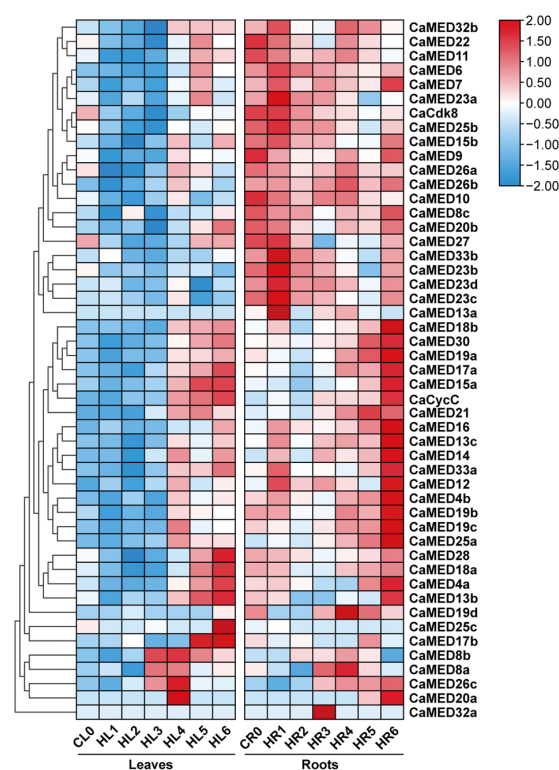


Figure 6. Expression patterns of pepper *CaMED* genes under heat stress. The heatmap was generated from PepperHub RNA-seq data to show *CaMED* transcript changes under heat treatment. The heat stress treatment time points were 1, 1.5, 3, 6, 12, and 24 h.

3.7. RT-qPCR Validation of Heat-Responsive *CaMED* Genes and Subcellular Localization of *CaMED25a*

To validate the transcriptome-based expression patterns and identify key *CaMED* genes associated with heat-stress responses, six candidate genes induced in both leaves and roots according to transcriptome analysis were selected for RT-qPCR validation. Leaf samples were collected after 42 °C treatment for 1, 2, 3, 4, 5, and 6 h (Figure 7A). The results showed that *CaMED4a*, *CaMED15a*, *CaMED19a*, *CaMED19c*, *CaMED21*, and *CaMED25a* were induced to varying degrees after heat treatment, and their expression trends were generally consistent with the transcriptome data. Although the transcriptome data were derived from publicly available datasets generated from a different pepper cultivar than that used for RT-qPCR validation, the two approaches showed broadly consistent expression trends, suggesting that these candidate genes exhibit relatively stable responses to heat stress. Among these genes, *CaMED25a* showed significant induction at several heat-treatment time points, indicating a strong transcriptional response to heat stress. Therefore, *CaMED25a* was chosen as a candidate gene for subsequent functional analyses.

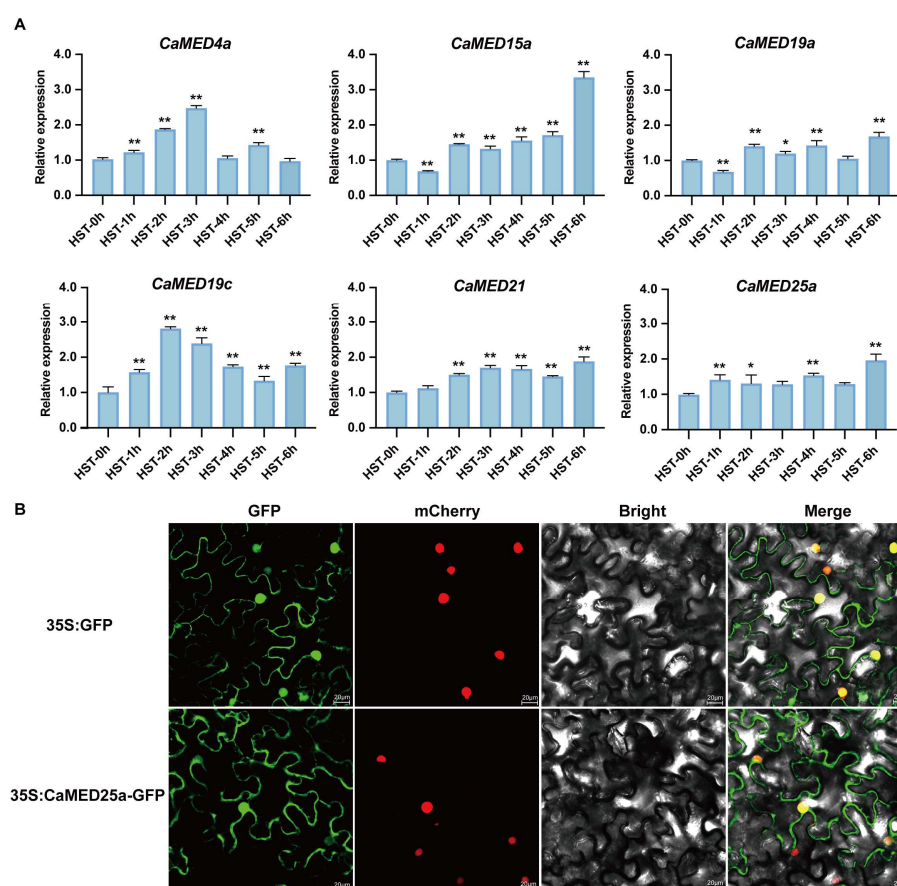


Figure 7. Relative expression analysis of selected *CaMED* genes under heat stress and subcellular localization of *CaMED25a*. (A) Relative transcript levels of selected *CaMED* genes in pepper leaves exposed to 42 °C for 1, 2, 3, 4, 5, and 6 h, as determined by RT-qPCR. Data represent the mean $\pm$ SD ($n = 3$). Different asterisks denote significant differences between heat-treated samples and the untreated control (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA with Tukey's test). (B) Subcellular localization of *CaMED25a* in leaf epidermal cells of *Nicotiana benthamiana*. H2B-mCherry was used as a nuclear marker, and 35S: GFP served as a positive control. Green fluorescence corresponds to GFP, red fluorescence corresponds to mCherry, and the yellow signal in the merged images reflects the overlap of the two fluorescent proteins. Scale bar = 20 μ m.

To gain insight into its potential biological function and determine whether it is localized in cellular compartments associated with transcriptional regulation, the subcellular localization of CaMED25a was further analyzed. The 35S: CaMED25a-GFP fusion protein produced clear fluorescence signals in both the nucleus and plasma membrane of *Nicotiana benthamiana* leaf cells (Figure 7B), indicating that CaMED25a is localized to the nucleus and plasma membrane.

3.8. Effects of Transient CaMED25a Silencing on Heat Stress Tolerance in Pepper

Based on the transcriptome analysis and RT-qPCR validation described above, *CaMED25a* was selected for further functional characterization because it displayed a strong and relatively stable response to heat stress. To determine whether *CaMED25a* contributes to pepper heat-stress tolerance, its expression was transiently suppressed using virus-induced gene silencing (VIGS). Silenced plants and corresponding controls at the same developmental stage were subsequently exposed to 42 °C. Compared with the TRV2:00 control, TRV2:*CaMED25a* plants exhibited more severe injury symptoms after heat stress treatment, indicating enhanced heat sensitivity (Figure 8A). RT-qPCR analysis confirmed that the transcript abundance of *CaMED25a* was significantly lower in TRV2:*CaMED25a* plants than in TRV2:00 plants, demonstrating effective gene silencing (Figure 8B). Further analysis showed that H₂O₂ accumulation was markedly increased in the leaves of TRV2:*CaMED25a* plants after heat stress (Figure 8C). Meanwhile, the heat defense-related genes *CaHSP18*, *CaHSP25.9*, and *CaHSP70.1* showed suppressed induction under heat stress (Figure 8D). These results indicate that silencing *CaMED25a* reduces heat tolerance in pepper, supporting its positive role in regulating heat-stress responses.

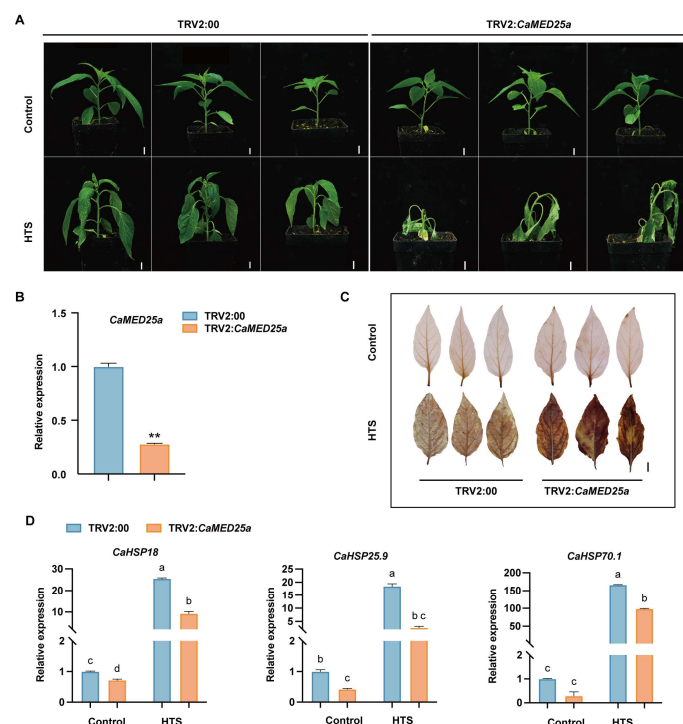


Figure 8. *CaMED25a* positively regulates heat tolerance in pepper. (A) Phenotypic comparison of TRV2:00 and TRV2:*CaMED25a* plants subjected to 42 °C heat stress. Scale bar = 1 cm. (B) Relative *CaMED25a* transcript levels in leaves of TRV2:00 and TRV2:*CaMED25a* plants. Data represent the mean $\pm$ SD ($n = 3$). ** $p < 0.01$ (Student's t -test). (C) DAB staining showing H₂O₂ accumulation in leaves of TRV2:00 and TRV2:*CaMED25a* plants. Scale bar = 1 cm. (D) Expression levels of heat-defense-related genes, including *CaHSP18*, *CaHSP25.9*, and *CaHSP70.1*, in pepper leaves before and after 42 °C treatment. Data represent the mean $\pm$ SD ($n = 3$). Different lowercase letters denote significant differences ($p < 0.05$, one-way ANOVA with Tukey's test).

4. Discussion

The Mediator complex acts as an important transcriptional co-regulator in plants and participates in a wide range of biological processes, including organ development, hormone responses, and stress responses [43,44]. Although genome-wide identification and characterization of *MED* genes have been reported in several plant species, such as Arabidopsis, rice, and tomato [23,24], information on this gene family in pepper remains limited. As an important horticultural crop, pepper is sensitive to heat stress, which can adversely affect plant growth, development, and yield formation [45]. In the present study, the pepper *CaMED* gene family was identified at the genome-wide level for the first time. Its family characteristics, promoter *cis*-acting elements, tissue-specific expression patterns, and heat stress-responsive transcriptional profiles were comprehensively analyzed to preliminarily insights into the potential roles of *CaMED* genes in pepper development and heat stress responses.

4.1. Characteristics and Evolutionary Analysis of the *CaMED* Gene Family

The Mediator complex is highly conserved during eukaryotic evolution and consists of multiple functionally specialized subunits [46]. To date, 54, 55, and 46 genes encoding *MED* subunits have been reported in rice, Arabidopsis, and tomato, respectively [23,24]. In this study, 49 *CaMED* genes were identified in pepper, and their modular classification was largely consistent with that reported in other plant species. These findings indicate that the general composition of the pepper *MED* family has remained relatively conserved, despite interspecific differences in the number of *MED* members.

In terms of structural characteristics, *CaMED* genes showed considerable differences in exon number, protein length, and theoretical isoelectric point, indicating structural diversity within this gene family. Comparable patterns have also been observed in *MED* gene families of tomato, cassava, and asparagus bean, where different members show substantial variation in exon–intron organization, protein length, and molecular weight [24,47,48]. These results further suggest that although the *MED* gene family maintains a relatively stable core composition, its members have undergone structural divergence to varying degrees.

Phylogenetic analysis showed that most *MED* proteins from pepper, Arabidopsis, and tomato clustered with their corresponding homologous clades (Figure 2), indicating a high degree of homology and evolutionary conservation among *MED* proteins across species, which is consistent with previous reports [23]. However, some members belonging to the same module were phylogenetically distant from one another (Figure 2), suggesting that partial sequence divergence has occurred among specific *MED* members. Duplication and synteny analyses identified only five pairs of intraspecific syntenic *CaMED* genes in pepper (Figure 3). In addition, dispersed duplication and singleton genes represented the predominant duplication categories, suggesting that the expansion of the *CaMED* family was not primarily driven by large-scale duplication events. Interspecific synteny analysis further indicated that the *CaMED* family is more conserved between closely related Solanaceae species. Overall, the *CaMED* family is evolutionarily conserved but exhibits partial divergence, suggesting that different *CaMED* members may have distinct roles in pepper growth, development, and stress responses.

4.2. Expression Characteristics and Functional Divergence of *CaMED* Genes

Promoter *cis*-element profiling is useful for inferring gene function from the perspective of transcriptional regulation. In this study, *CaMED* family members contained abundant elements associated with light response, phytohormone signaling, and stress regulation. Notably, light-responsive elements were identified in all *CaMED* promoters, indicating their potential responsiveness to light-related signals. Previous studies have

shown that MED subunits participate in light-associated regulatory pathways. For example, *Arabidopsis* MED25/PFT1 regulates FT expression through the phytochrome signaling pathway [49,50]. MED18 also cooperates with HY5 to regulate anthocyanin accumulation under high light conditions [51]. These studies provide a useful framework for investigating the potential roles of *CaMED* genes in light-responsive processes in pepper. In addition, *CaMED* promoters commonly contained phytohormone-responsive elements related to ABA, MeJA, IAA, and GA, as well as stress-responsive elements associated with drought and other stresses. Previous reports have demonstrated that MED subunits participate broadly in hormone- and stress-related regulatory processes. For example, RhMED15A participates in drought stress responses in rose through ABA- and MeJA-related pathways [52]. In this study, the promoters of *CaMED15a* and *CaMED15b* were also enriched in ABA-responsive, MeJA-responsive, and stress-related elements, suggesting that they may have similar functions in pepper. Overall, the cis-element composition of *CaMED* promoters suggests potential roles in growth, development, and stress responses in pepper; however, these predictions require further experimental validation.

Tissue expression profiling further revealed distinct expression patterns of *CaMED* family members across different organs and developmental stages in pepper. Most *CaMED* members were highly expressed during early flower development and late placenta development, suggesting that this gene family may be closely associated with reproductive organ development in pepper. Previous reports indicate that MED18 participates in floral organ formation and flowering-time control in *Arabidopsis* [53]. MED25/PFT1 negatively regulates floral organ size by modulating cell proliferation and cell expansion [54]. These findings provide a useful framework for exploring the potential roles of *CaMED* genes in reproductive development. Furthermore, *CaMED8a* showed relatively high expression in leaves, and MED8 has been reported to regulate leaf thickness and leaf number in tobacco [55], suggesting that *CaMED8a* may have a similar role in vegetative organ development in pepper. Taken together, the differential expression patterns of *CaMED* family members in different tissues suggest that they may perform distinct biological functions during pepper growth and development.

Heat stress is an important environmental constraint affecting pepper growth, development, and yield formation. Therefore, analyzing heat-responsive patterns at the gene family level is important for identifying key regulatory factors and elucidating the molecular mechanisms underlying heat tolerance. Previous studies have shown that Mediator subunits participate in both heat-stress responses and thermomorphogenesis in plants. In *Arabidopsis*, MED14 and MED17 participate in heat-induced transcriptional reprogramming and act as coactivators of heat-induced gene expression, and their mutants usually exhibit heat-sensitive phenotypes [19]. MED14 also cooperates with PIF4 to regulate hypocotyl elongation under high temperature conditions [56]. In the present study, the pepper *CaMED* family also showed clear responses to heat stress, but the response patterns differed among tissues. In leaves, most *CaMED* members were mainly upregulated after heat treatment, whereas both upregulated and downregulated patterns were observed in roots. The generally consistent expression trends obtained from public transcriptome datasets and RT-qPCR validation suggest that several *CaMED* genes may participate in pepper heat-stress responses. Notably, MED25 family members have been reported to play important roles in plant responses to environmental stimuli and abiotic stresses [57,58]. Similar to conclusions reported in previous studies, *CaMED25a*, which may also participate in abiotic stress responses, exhibited a pronounced heat-responsive expression pattern, supporting its potential involvement in regulating heat-stress responses in pepper.

4.3. *CaMED25a* Participates in Heat Stress Tolerance Regulation in Pepper

Based on the heat stress expression profiles and RT-qPCR validation results described above, *CaMED25a* was selected as a key candidate gene involved in the pepper heat stress response. Functional validation using VIGS showed that silencing *CaMED25a* increased heat sensitivity in pepper plants under high-temperature conditions, indicating that *CaMED25a* participates in the regulation of heat stress tolerance. Previous studies have shown that *Arabidopsis* MED25 promotes *YUCCA8* transcription under high-temperature conditions by interacting with PIF4 and HDA9, thereby promoting hypocotyl elongation [58]. In contrast to previous reports in which MED25 was mainly associated with thermomorphogenesis, our results indicate that *CaMED25a* contributes importantly to heat stress tolerance in pepper. This finding indicates that MED25 homologs may not only participate in temperature-induced morphological regulation but also contribute to plant adaptation to heat stress, suggesting functional divergence in different plant backgrounds.

Heat stress generally induces excessive production of reactive oxygen species (ROS), which may subsequently cause cellular injury. Previous studies have shown that *CaWRKY27* regulates heat tolerance in pepper through an H_2O_2 -related pathway, further supporting the importance of ROS homeostasis in pepper heat stress responses [59]. In this study, DAB staining showed that H_2O_2 accumulation was markedly increased in *CaMED25a*-silenced plants after heat stress, consistent with their heat-sensitive phenotype. Meanwhile, the heat-induced transcription of heat defense-related genes, including *CaHSP18*, *CaHSP25.9*, and *CaHSP70.1*, was inhibited in *CaMED25a*-silenced plants. Previous studies have shown that pepper HSP family members are involved in heat stress responses and contribute to protein homeostasis and thermotolerance [60–62]. Therefore, *CaMED25a* may improve heat stress tolerance in pepper by maintaining ROS homeostasis and promoting the expression of heat defense-related genes. However, the current results do not determine whether *CaMED25a* directly regulates the expression of *CaHSP18*, *CaHSP25.9*, and *CaHSP70.1*. Given that Mediator subunits usually function as transcriptional coactivators by interacting with specific transcription factors to regulate gene transcription [4], we hypothesize that *CaMED25a* may enhance pepper thermotolerance through interactions with heat-responsive transcription factors, such as HSFs, thereby facilitating the activation of downstream heat defense genes and ROS-scavenging pathways. This hypothesis is supported by the reduced expression of heat-responsive genes and increased H_2O_2 accumulation observed in *CaMED25a*-silenced plants. Future studies combining protein–protein interaction analyses, transcriptome profiling, and target-gene identification will help test this hypothesis and elucidate the molecular mechanism underlying heat-stress responses mediated by the *CaMED25a* protein in pepper.

5. Conclusions

In the present study, the pepper *CaMED* gene family was identified and systematically characterized at the genome-wide level for the first time. A total of 49 *CaMED* members were obtained, and their chromosomal localization, gene organization, physicochemical characteristics, phylogenetic relationships, syntenic associations, promoter cis-acting elements, tissue expression patterns, and heat-responsive features were comprehensively analyzed using publicly available genomic and transcriptomic datasets. The results showed that the *CaMED* family is highly conserved during evolution, while different members exhibit clear divergence in structural features, regulatory element composition, and expression patterns. Expression analysis indicated that the *CaMED* family may participate broadly in pepper growth, development, and heat stress responses. Furthermore, experimental analyses, including RT-qPCR expression profiling and VIGS-mediated functional validation, demonstrated that *CaMED25a* is involved in regulating heat stress tolerance in

pepper. Silencing *CaMED25a* decreased heat tolerance, enhanced H₂O₂ accumulation, and suppressed the transcription of heat defense-related genes. Overall, this study provides an important reference for understanding the composition, evolutionary relationships, and functional divergence of the pepper *CaMED* gene family, while also offering a theoretical basis for future studies on the molecular regulation of heat stress responses in pepper.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070772/s1>, Figure S1: Gene structure analysis of pepper *CaMED* genes; Table S1: Primer information used in this study; Table S2: Summary of *CaMED* gene information in pepper; Table S3: Duplication types and intragenomic duplicated gene pairs of the *CaMED* family.

Author Contributions: Conceptualization, methodology, data curation, and writing—original draft preparation, H.Y.; methodology and investigation, B.T. and Z.J.; formal analysis, Q.Y.; data curation, J.D.; funding acquisition and resources, C.X.; project administration, supervision, funding acquisition, and writing—review and editing, F.L. and X.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the National Natural Science Foundation of China (Grant Nos. 32341047 and U22A20497).

Data Availability Statement: The data supporting the findings of this study are available within the article and its Supplementary Materials. Additional relevant data are available from the corresponding author upon reasonable request, subject to the data management and authorization rules of the corresponding author's institution.

Acknowledgments: The authors thank the Hunan Agricultural University Pepper Research Team for providing the pepper material ‘Zhangshugang’ used in this study. The authors also acknowledge PepperHub for providing publicly available genomic and transcriptomic resources for pepper research. During the preparation of this manuscript, the authors used ChatGPT (OpenAI, GPT-5.5 Thinking) for language polishing. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

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

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