Characterization and Functional Analysis of Calmodulin and Calmodulin-Like Genes in *Fragaria vesca*

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Calcium is a universal messenger that is involved in the modulation of diverse developmental and adaptive processes in response to various stimuli. Calmodulin (CaM) and calmodulin-like (CML) proteins are major calcium sensors in all eukaryotes, and they have been extensively investigated for many years in plants and animals. However, little is known about CaMs and CMLs in woodland strawberry (*Fragaria vesca*). In this study, we performed a genome-wide analysis of the strawberry genome and identified 4 CaM and 36 CML genes. Bioinformatics analyses, including gene structure, phylogenetic tree, syntenic and three-dimensional model assessments, revealed the conservation and divergence of *FvCaMs* and *FvCMLs*, thus providing insight regarding their functions. In addition, the transcript abundance of four *FvCaM* genes and the four most related *FvCML* genes were examined in different tissues and in response to multiple stress and hormone treatments. Moreover, we investigated the subcellular localization of several *FvCaMs* and *FvCMLs*, revealing their potential interactions based on the localizations and potential functions. Furthermore, overexpression of five *FvCaM* and *FvCML* genes could not induce a hypersensitive response, but four of the five genes could increase resistance to *Agrobacterium tumefaciens* in *Nicotiana benthamiana* leaves. This study provides evidence for the biological roles of *FvCaM* and *CML* genes, and the results lay the foundation for future functional studies of these genes.

**Keywords:** strawberry (*Fragaria vesca*), bioinformatics analysis, expression profile, subcellular localization, transient assay

**INTRODUCTION**

Ca²⁺, which functions as a universal second messenger, plays vital roles in the developmental processes of plants and in response to various environmental stimuli (Trewavas and Malho, 1998; Berridge et al., 2000). Plants have evolved a diversity of unique proteins that bind to Ca²⁺, most commonly via the evolutionarily conserved EF-hand motif (Clapham, 2007). This conserved motif
has been extensively studied (Kawasaki et al., 1998; Gifford et al., 2007). More than 250 proteins containing EF-hands have been found in the Arabidopsis genome (Day et al., 2002). CaM, as well as CML, one of the most well described Ca2+ sensors, is composed of EF-hands and is essential for calcium signal transduction in plants.

Calmodulins are highly conserved among eukaryotic proteins, exhibiting almost 90% identity in humans and plants. In contrast, CMLs are restricted to plants (DeFalco et al., 2010). CaMs are encoded by only a few CaM genes in animal genomes (e.g., three in humans), whereas plant genomes carry a greater number of CaM genes and multiple loci containing identical CaM genes (DeFalco et al., 2010). In plant genomes, the CaM/CML gene families are represented by seven CaM genes, including two sets of isoforms encoding identical proteins (e.g., CaM1 and CaM4; CaM2, CaM3, and CaM5), 50 CML genes in Arabidopsis (McCormack and Braam, 2003), and five CaM genes and 32 CML genes in rice (Boonburapong and Buaboocha, 2007).

The conservation of CaMs reflects the strict restrictions on the structure of the proteins. A typical CaM contains four EF-hand motifs (Snedden and Fromm, 2001), but for CMLs, this number ranges from one to six, and some of the motifs are degraded (McCormack and Braam, 2003; Boonburapong and Buaboocha, 2007). Structurally, the EF-hand motif is composed of two helices, the E helix and the F helix, flanking a Ca2+-binding loop in a structure that resembles a hand (McCormack et al., 2005). The crystal structure of CaMs has revealed that these proteins comprise two globular domains (the N-lobe and the C-lobe) (Babu et al., 1984), each containing a pair of EF-hands with affinity for Ca2+ (Tidow and Nissen, 2013). The binding of Ca2+ to CaMs induces a conformational change that results in exposure of the hydrophobic surface, which then interacts with downstream CaM-binding proteins (CBPs) (Kursula, 2014).

As essential Ca2+ sensors, CaMs are involved in a series of developmental processes, especially in stress-induced signaling pathways. Accumulating evidence indicates that gene overexpression or loss of function in plants carrying mutations in CaM and CML genes strongly affects the immune system. For example, down-regulation of the pathogen-induced CaM genes, NtCaM1 and NtCaM13, was found to differentially impact disease resistance in tobacco (Takahatake et al., 2007). The loss-of-function cml9 mutation in Arabidopsis was shown to enhance susceptibility to pathogens, and overexpression of AtCML9 reduced susceptibility to virulent strains of Pst (Magnan et al., 2008). In addition to biotic stress, CaMs and CMLs are also involved in abiotic stress responses. GmtCaM4, a salt-stress induced gene, has been reported to mediate Ca2+ signaling and the downstream transcription factor (TF), MYB2 (Rao et al., 2014). In addition, a CaM3 knockout Arabidopsis mutant displayed impaired thermotolerance, whereas overexpression of AtCaM3 increased plant thermotolerance, via interactions with heat stress transcription factors (HSFs) (Zhang et al., 2009). Moreover, a series of studies have demonstrated the roles of CaMs and CMLs in hormone signaling (Kaplan et al., 2006; Du et al., 2009).

To date, no systematic research has been conducted to investigate CaM and CML genes in strawberry. In the present study, we performed a genome-wide analysis and identified 4 CaM genes and 36 CML genes in the woodland strawberry genome (Fragaria vesca). The gene structure analysis suggested that FvCaMs possess a conserved motif organization, while FvCMLs possess complex structures in terms of both motif organization and exon–intron structure. Phylogenetic tree and synteny analyses provided insight regarding the evolution and function of FvCaM and FvCML genes. Using AtCaM7 as template, we predicted the three-dimensional structure of FvCaM1, thus facilitating our understanding of the mechanisms of FvCaMs. In addition, we assessed the transcript abundance in different tissues and in response to multiple stress and hormone treatments. Furthermore, we investigated the subcellular localization of several FvCaMs and CMLs in Arabidopsis mesophyll protoplasts under normal condition or in response to diverse treatments. We also performed transient assays to explore the biological roles of FvCaMs and FvCMLs in Nicotiana benthamiana leaves. Our results provide a subset of FvCaM and CML genes that could be targeted in future engineering strategies to modify both pathogen resistance and stress tolerance in strawberry.

**MATERIALS AND METHODS**

**Identification of CaM and CML Genes in the Strawberry Genome**

To identify CaM and CML homologs in the strawberry genome (F. vesca), we performed a Blast-P search against the RefSeq protein database of F. vesca in NCBI with the default parameters. The amino acid sequence of AtCaM1 gene was chosen as the query. After that, we manually chose the proteins with the sequence identity of more than 85 and 16% as putative CaMs and CMLs, respectively. All of the putative candidates were manually verified using the online software InterProScan1) and Pfam2) to check their completeness and to confirm the presence of the EF-hand domain. The phylogenetic relationships of the candidates were determined to confirm that they were FvCaMs or FvCML members. The corresponding nucleotide sequences as well as information regarding each gene were obtained from NCBI. The sequences of Arabidopsis and rice CaM and CML genes were obtained from the Arabidopsis Information Resource (TAIR3) and rice genome database in TIGR4, respectively.

**Evolutionary and Structural Analyses**

The amino acid sequences of CaMs and CMLs from Arabidopsis, rice and strawberry were aligned using ClustalX with default settings. The phylogenetic tree was constructed with MEGA5.0 using the neighbor-joining method and a bootstrap setting of up to 1000 replicates. The organization of the FvCaM and FvCML

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1) http://www.ebi.ac.uk/interpro/search/sequence-search
2) http://pfam.xfam.org
3) http://www.Arabidopsis.org
4) http://rice.tigr.org
motifs was derived using the online tool MEME\(^5\) and visualized by DOG 2.0.\(^6\) Analysis of the exon–intron structure was conducted using GSDS 2.0\(^7\) by aligning the cDNA sequences with their corresponding genomic DNA sequences. Synteny blocks between *Arabidopsis* and strawberry genomes were obtained from the Plant Genome Duplication Database\(^8\). All of the identified relevant CaM and CML genes were visualized using Circos (Krzywinski et al., 2009) and Adobe Photoshop CS6.

The FvCaMs amino acid sequences were aligned with AtCaM7 using ClustalX version 2.0.12 and embellished using GeneDoc. The three-dimensional structure was predicted and visualized by Swiss-Model\(^9\) using *Arabidopsis* AtCaM7 as the template (Tidow et al., 2012). The structural details and annotations are presented in reference to a previously published paper (McCormack et al., 2005).

### Plant Materials and Treatments

The Chinese woodland strawberry, *F. vesca* accession Heilongjiang-3, was grown in the greenhouse at Northwest A\&F University in China at a temperature of 18–23°C, relative humidity of 60–80%, and without supplemental lighting. Three-month-old strawberry seedlings, at which time the sixth leaf was fully expanded, were selected for the treatments. *Arabidopsis thaliana* ecotype Col-0 plants were grown at 22°C with a relative humidity of 75% and under short-day (8 h of light at 125 \(\mu\)mol·m\(^{-2}\)·s\(^{-1}\), 16 h of dark) conditions for 4–5 weeks before transformation.

The PM treatment experiment was conducted by touching the adaxial epidermis of the strawberry leaves with sporulating colonies. Treated plants were then incubated under controlled conditions. Inoculated leaves were collected at 0, 24, 48, 72, 96, 120, 144, and 168 h post-inoculation (hpi). Drought stress treatment was performed by withholding water followed by sampling at 0, 24, 48, 96, 120, 144, and 168 h post-treatment (hpt). Plants were recovered after 168 h of drought stress by watering and sampled after an additional 24 h. Salt stress treatment was applied to 3-month-old substrate-grown strawberry seedlings via irrigation with 300 mM NaCl. Treated leaves were sampled at 0, 0.5, 2, 4, 8, 12, 24, and 48 hpt. Heat stress treatment was applied by transferring the seedlings to 42°C, and the treated leaves were collected at 0, 0.5, 2, 4, 8, 12, 24, and 48 hpt. For the hormone treatments, the strawberry leaves were sprayed with 1 mM ABA, 1 mM SA, 0.1 mM MeJA, or 0.5 g/L Eth, and the leaves were collected at 0, 0.5, 2, 4, 8, 12, 24, and 48 hpt for RNA isolation. All of the plants were treated in the light, and three independent experiments were performed.

### Semi-Quantitative Reverse-Transcription PCR

Total RNA was extracted from treated strawberry leaves and different tissues exposed to normal conditions using the E.Z.N.A. Plant RNA Kit (Omega, Guangzhou, China) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 2 \(\mu\)g of total RNA using PrimeScript RTase (Takara, Dalian, China). The concentration of cDNA was adjusted according to the internal reference gene *Fv18S* (GenBank accession: XM_011464048). We also use two additional internal reference genes, *FvGAPDH1* (GenBank accession: XM_004306515) and *FvGAPDH2* (GenBank accession: XM_004309993) to evaluate the quality and consistence of the cDNA (Francisco et al., 2013). RT-PCR reactions were conducted using the following profile: initial denaturation at 94°C for 2 min, followed by 30–36 cycles of denaturation at 92°C for 30 s, annealing at 60 ± 3°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 2 min. PCR products were separated in a 1.5% (w/v) agarose gel stained with ethidium bromide and imaged under UV light for further gene expression analysis. Each reaction was repeated three times, and the three independent analyses revealed the same trends for each gene and treatment. Expression levels measured by semi-quantitative PCR were quantified as the fold-change in response to the experimental treatments relative to the control samples and are presented as heat maps that were generated using GeneSnap and Mev 4.8.1. The primers used for RT-PCR are listed in Supplementary Table S1.

### Plasmid Construction

The predicted full-length coding sequences of *FvCaMs* and *FvCMLs*, including *FvCaM2*, *CaM3* and *CML15*, and *CML28*, were amplified using high-fidelity Taq HS-mediated PCR from the cDNA of woodland strawberry *F. vesca* accession Heilongjiang-3 leaves. The amplified PCR products were cloned into the pBI221 vector (Clontech, Beijing, China) containing the *CaMV 35S* promoter and GFP using the ClonExpress II One Step Cloning Kit (Vazyme, USA), resulting in plasmids pFvCaMs-GFP and pFvCMLs-GFP. The elicitor protein gene, INF1, was cloned as previously reported (Kamoun et al., 1998). Plasmid pINF1-GFP was constructed as described above. The primers used for cloning genes and constructing vectors are shown in Supplementary Table S1.

### Protoplast Isolation, Transfection, and Treatment

*Arabidopsis* mesophyll protoplasts were isolated from 4-week-old Col-0 leaves using a previously described method (Yoo et al., 2007). The recombinant plasmids and control plasmid (pBI221) were isolated using the Omega Plasmid Mini Kit (Omega, Guangzhou, USA). The DNA concentration was adjusted to 500 ng·L\(^{-1}\) per 5 kb of DNA. For transfection, 200 \(\mu\)L of *Arabidopsis* mesophyll protoplast was transferred into a 2-\(\mu\)L round-bottom microcentrifuge tube, in which 200 \(\mu\)L of protoplast solution was mixed with 10 \(\mu\)L of recombinant or control plasmid. The transfection procedure was performed as previously described (Yoo et al., 2007). After transfection, *Arabidopsis* protoplasts were incubated in the dark at room temperature for 16–18 h before examination by fluorescence microscopy. Images were acquired using an Olympus BX-51 inverted fluorescence microscope (Olympus, Japan). The imaged
data were processed using Adobe Photoshop (Mountain View, CA, USA). For multiple treatments, *Arabidopsis* protoplasts were treated for a duration of 16–18 h with 50 µM H_2O_2, 100 µM SA, 100 µM MeJA or 20 µM Eth after transfection and then examined using fluorescence microscopy. For the 4 and 42°C treatments, *Arabidopsis* protoplasts were first incubated at room temperature for 12 hpt and then transferred into a 4 or 42°C chamber for another 4 h. All of the experiments were repeated three times.

### Transient Expression Assay in *N. benthamiana* Leaves

*Agrobacterium tumefaciens* strain GV3101 individual and GV3101 transformed with pBI221, pFvCaMs-GFP, pFvCMLs-GFP, or pINF1-GFP were grown overnight in LB medium supplemented with 50 mg/L of rifampicin, 100 mg/L of ampicillin and 25 mg/L of gentamicin. The *Agrobacterium* suspensions were centrifuged and resuspended in infiltration buffer (10 mM MgCl_2, 10 mM MES, and 100 µM acetylsheringone). The suspensions were adjusted to an OD_600 of 0.5 and maintained at room temperature for more than 3 h. The bacterial suspensions were infiltrated into the leaves of 4-week-old *N. benthamiana* using a syringe without a needle. Plants infiltrated with *A. tumefaciens* were grown in a greenhouse at 23°C. For the transient assay of individual genes, we first injected each 100 µL of *Agrobacterium* suspension with pFvCaM-GFP, pFvCML-GFP, or pBI221 plasmid (as a control), and 24 h post-transformation, we injected 10 mM CaCl_2, 10 mM EGTA or infiltration buffer solution (as a control) at the corresponding sites. The experiments were repeated three times with equivalent results. For the HR analysis, we first injected each 100 µL of *Agrobacterium* suspensions with pFvCaM-GFP, pFvCML-GFP, and pBI221 plasmid (as a control), and at 24 h post-transformation, we injected another 100 µL of each *Agrobacterium* suspension containing pINF1-GFP or GV3101 alone. The experiments were repeated three times with similar results.

### RESULTS

#### Characteristics of *CaM* and *CML* Genes in Strawberry (*Fragaria vesca*)

To identify *CaM* and *CML* genes in the strawberry genome, we first determined the amino acid sequence of the *AtCaM1* gene of *Arabidopsis*. Next, we performed a Blast-P search against the strawberry genome (*F. vesca*) in NCBI using the amino acid sequence of *AtCaM1* as the query. Protein sequences with an identity of more than 85% to *AtCaM1* were defined as FvCaMs, and those with an identity of more than 16% were defined as FvCMLs. Preliminarily, we identified 8 FvCaM genes and 49 FvCML genes (data not shown). Further testing indicated that four (LOC101297640, LOC101308034, LOC101303028, and LOC101303236) of the eight FvCaM genes belonged to the CML group (*Figures 1A* and 2), and some of the FvCMLs were recognized as CBLS and CDPKs. By removing the incomplete genes, transcripts of the same genes and redundant sequences, a total of 4 FvCaM genes and 36 FvCML genes were identified (*Table 1*). The putative protein sequences corresponding to the *CaM* and *CML* genes were submitted to Pfam and InterproScan to confirm the presence of the EF-hand domain and the absence of other functional domains. It is noteworthy that FvCaM1, which displayed a high level of homology to OsCML1, is still considered a *CaM* gene because it exhibits a high similarity (more than 90%) to *AtCaM1* and contains a typical CaM structure with four conserved EF-hands; a similar finding was obtained for FvCaM4. By contrast, FvCML24 is closely related to CaMs phylogenetically, however, it lacks the typical EF-hand domain. Thus, we excluded it from the CaM group and defined it as a CML gene.

The putative 4 FvCaM genes and 36 FvCML genes were renamed based on their chromosome distributions. A series of correlative information for the FvCaMs and FvCMLs is shown in *Table 1*. The four FvCaM genes were distributed on chromosome Numbers 1 and 7, while the 36 FvCML genes were distributed across all seven chromosomes, excluding FvCML36, the location of which is still unknown in the strawberry genome. The FvCaM gene family contains four members, each of which is predicted to have four EF-hands. In contrast, the number of EF-hands in FvCMLs varies from two to four. The 4 FvCaMs and 36 FvCMLs were grouped into five subgroups, as shown in *Figure 1*.

#### Phylogenetic Relationships of *CaM* and *CML* Genes among *Arabidopsis*, Rice and Strawberry

To investigate the evolution and to gain some insight into the potential functions of FvCaMs and FvCMLs, we constructed an unrooted phylogenetic tree using the full-length amino acid sequences from *Arabidopsis*, rice and strawberry. As shown in *Figure 2*, CaMs and CMLs were clearly separated but still displayed close phylogenetic relationships. FvCaMs clustered with AtCaMs and OsCaMs, with the one exception of OsCML1, which clustered with FvCaM1. In addition to CaMs, the CMLs clades were well defined. The CMLs from *Arabidopsis*, rice and strawberry clustered into four distinct clades, which were defined as subgroups 2 to 5 based on the orders. Subgroup 2 members displayed a high sequence similarity with CaMs and consisted of 13 FvCML members. Among them, FvCML24 showed the closest relationship with CaMs, supporting its potential function as a CaM. Subgroup 3 contained 11 FvCML members, most of which clustered with AtCMLs, excluding FvCML26, which showed a close phylogenetic relationship with OsCML25. In addition, nine FvCMLs were grouped in subgroup 4 and three in subgroup 5. Intriguingly, no OsCML was found in subgroup 5. Taken together, these findings showed that FvCaMs and FvCMLs clustered more frequently with *Arabidopsis* CaMs and CMLs than with rice.

#### The Gene Structures of *FvCaMs* and *FvCMLs*

The gene structures were found to be relatively conserved among FvCaMs but variable among FvCMLs. The four FvCaM genes each contained the typical four EF-hands (*Figure 1B*), each of which had the ability to bind calcium ions. Motifs 1, 2, and 3...
TABLE 1 | Characterizations of FvCaMs and FvCMLs.

| Name       | Gene symbol1 | Chromosome | Genomic location       | Number of EF-hands2 | Subgroup3 |
|------------|--------------|------------|------------------------|---------------------|-----------|
| FvCaM1     | LOC101310634 | 1          | 1453771..1454591       | 4                   | 1         |
| FvCaM2     | LOC101306277 | 1          | 21925486..21927165     | 4                   | 1         |
| FvCaM3     | LOC101308034 | 7          | 15905182..15905769     | 4                   | 1         |
| FvCaM4     | LOC101308330 | 7          | 15907251..15908052     | 4                   | 1         |
| FvCML1     | LOC101311505 | 1          | 28302329..28314148     | 4                   | 4         |
| FvCML2     | LOC101295453 | 1          | 10796020..10797900     | 4                   | 2         |
| FvCML3     | LOC101296330 | 1          | 11096586..11097694     | 4                   | 3         |
| FvCML4     | LOC101299034 | 1          | 15734366..15743802     | 4                   | 3         |
| FvCML5     | LOC101294531 | 2          | 5986641..5987270       | 4                   | 5         |
| FvCML6     | LOC101294415 | 2          | 8519239..8520134       | 4                   | 4         |
| FvCML7     | LOC101309601 | 2          | 16209859..162011260    | 4                   | 2         |
| FvCML8     | LOC101311830 | 2          | 19404646..19405242     | 3                   | 4         |
| FvCML9     | LOC101314014 | 2          | 22148679..22149342     | 2                   | 4         |
| FvCML10    | LOC101314014 | 2          | 32740741..32741328     | 3                   | 3         |
| FvCML11    | LOC101305440 | 3          | 2527146..25287736      | 2                   | 3         |
| FvCML12    | LOC101296846 | 3          | 21600013..21604084     | 4                   | 3         |
| FvCML13    | LOC101299799 | 3          | 22541413..22544136     | 4                   | 2         |
| FvCML14    | LOC101302252 | 3          | 26376949..26386401     | 2                   | 3         |
| FvCML15    | LOC101303028 | 4          | 591675..5943796        | 4                   | 2         |
| FvCML16    | LOC101313108 | 4          | 16571278..16572296     | 4                   | 5         |
| FvCML17    | LOC101300326 | 4          | 17899738..17902062     | 2                   | 4         |
| FvCML18    | LOC101304964 | 4          | 19239194..19239748     | 4                   | 3         |
| FvCML19    | LOC101292012 | 4          | 20443557..20444310     | 4                   | 3         |
| FvCML20    | LOC101292995 | 5          | 375772..376584         | 3                   | 2         |
| FvCML21    | LOC101302652 | 5          | 496258..496366         | 4                   | 2         |
| FvCML22    | LOC101297834 | 5          | 7131803..7132588       | 4                   | 5         |
| FvCML23    | LOC101300877 | 5          | 820887..8209177       | 2                    | 4         |
| FvCML24    | LOC101297640 | 5          | 7973286..7976512      | 4                    | 2         |
| FvCML25    | LOC101309155 | 5          | 10142011..10142805     | 4                    | 3         |
| FvCML26    | LOC101295526 | 5          | 24687301..24688015     | 4                    | 3         |
| FvCML27    | LOC101296206 | 5          | 26597114..26597991     | 3                    | 3         |
| FvCML28    | LOC101303236 | 6          | 507656..509481         | 4                    | 2         |
| FvCML29    | LOC101295524 | 6          | 628567..6286493       | 3                    | 4         |
| FvCML30    | LOC101307708 | 6          | 18470935..18471614     | 2                    | 3         |
| FvCML31    | LOC101298441 | 6          | 2317461..23175773      | 4                    | 2         |
| FvCML32    | LOC101306735 | 6          | 23523213..23525234     | 2                    | 3         |
| FvCML33    | LOC101313114 | 6          | 3763246..3764212       | 2                    | 4         |
| FvCML34    | LOC101298633 | 7          | 761066..7611796        | 4                    | 3         |
| FvCML35    | LOC101294800 | 7          | 20275502..20276323     | 4                    | 5         |
| FvCML36    | LOC101310856 | Un         | 165612..166327         | 4                    | 4         |

1Gene symbols are available in NCBI. 2The number of EF-hands was predicted using the MEME tool. 3These genes are grouped according to the phylogenetic tree.

represent three distinct types of EF-hand (Figure 1B). FvCaMs only contained motifs 1 and 2, which compose a typical EF-hand domain pair. FvCML subgroup 2 members exhibited the closest phylogenetic relationship to FvCaMs and possessed a similar motif organization as FvCaMs (Figure 1B). In particular, the most closely related genes, including FvCML24, CML28, and CML7, were in the same mode as FvCaMs (Figure 1B). Subgroup 3 and 4 members exhibited a more complex motif organization. However, they still contained the similar motif organization, and some of the subgroups contained the same motif organization, such as FvCML9, CML23, CML17, and CML33, each of which carried one each of motif 1 and motif 2 (Figure 1B). Interestingly, subgroup 5 members had distant phylogenetic relationships from other FvCMLs (Figure 1A), but these four genes possessed conserved motif organizations. Regarding exon–intron structure, the majority of the FvCaM and FvCML genes lacked introns (Figure 1C). However, some of the genes contained intron insertions that did not follow any rule and that varied in amount and intron phase. Regardless, most of the phylogenetically closely related genes possessed the same or similar exon–intron structures. Taken together, the motif organizations and exon–intron structures revealed...
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FIGURE 1 | Gene structure and phylogenetic relationship of FvCaM and FvCML genes. (A) Phylogenetic tree of FvCaMs and FvCMLs. The phylogenetic tree was constructed using the full-length protein sequences of FvCaMs and CMLs by the neighbor-joining method with 1,000 bootstrap replicates. (B) Motif organization of the FvCaM and FvCML genes. The three colored boxes represent three distinct EF-hand motifs. (C) Exon–intron structures of the FvCaM and FvCML genes. The exons, introns and UTRs are represented by blue boxes, fold lines and green boxes, respectively. The intron phases are annotated by 0, 1, and 2. The sizes of the exons are proportional to their sequence lengths. The FvCaMs and FvCMLs can be divided into five subgroups, which are denoted as subgroups 1–5 from top to bottom.

the conservation of gene structures. For example, FvCaM1 and CaM2, FvCML5, and CML35 possessed the same motifs and exon–intron organization (Figures 1B,C). Clustering of the motifs and exon–intron organization of the FvCaM and FvCML genes using an unrooted phylogenetic tree suggested an association between gene structures and evolutionary relationships.

Syntenny Analysis of CaM and CML Genes between Arabidopsis and Strawberry

To further study the origin and evolution of FvCaM and FvCML genes, we investigated the syntenic blocks between Arabidopsis and strawberry. In total, we found 24 pairs of syntenic genes containing FvCaMs or FvCMLs (data not shown). However, no syntenic relationship was observed between FvCaMs and AtCaMs. In addition, only 5 of the 24 pairs of syntenic genes were recognized as FvCML-AtCML gene pairs (Figure 3; Supplementary Table S2); and each pair of genes was phylogenetically closely related (Figure 2). It is worth noting that AtCML8 exhibited two syntenic CML genes (FvCML15 and FvCML28) in the strawberry genome, and FvCML28 had two syntenic CML genes (ATCML8 and AtCML11) in Arabidopsis (Figure 3). In addition, FvCML15 was found to be phylogenetically closely related to both AtCML8 and AtCML11 (Figure 2). These relationships are quite complex, but we can still speculate that AtCML8 and AtCML11 are duplicated genes that arose before the divergence of Arabidopsis and strawberry. FvCML28 and FvCML15 were found to be duplicated genes that arose after the divergence of the two species. In addition, FvCML27 and AtCML41 were closely related in the phylogenetic tree (Figure 2), supporting the evolution of a single process. These results provide insight facilitating further investigations of the functions of FvCML genes.
Multiple Alignment and Protein Structure of FvCaMs

Due to the similar sequences of CaMs among eukaryotic cells, the structures of CaMs are conserved. As shown in Figure 4C, the four FvCaMs aligned with Arabidopsis AtCaM7, the 3D structure of which has been reported (Tidow et al., 2012). The CaMs were composed of four EF-hand motifs, and each motif exhibited a helix-loop-helix structure (Figure 4A). FvCaM1 and FvCaM2 were extremely similar to AtCaM7, especially in terms of the EF-hand motifs. By contrast, FvCaM3 and FvCaM4, despite their variation in some amino acid sequences, were conserved at key sites in the EF-hand motif (Figure 4C). The canonical EF-hand motif of CaMs binds to Ca$^{2+}$ via pentagonal bipyramidal geometry consisting of six coordination sites, with the sixth position located in the loop (Kawasaki et al., 1998). The corresponding six sites, the 1st, 3rd, 5th, 7th, 9th, and 12th amino acids (alternatively denoted X, Y, Z, #, −X, −Z) possess the ability to chelate Ca$^{2+}$, as demonstrated by the 3D structure shown in Figure 4B (McCormack et al., 2005). The sixth position, which is usually a glycine (G), is thought to be important for the formation of the sharp turn within the loop. Notably, the seven sites involved in chelation are strongly conserved. The flanking E and F helices are generally composed of nine conserved amino acids with a regular distribution of hydrophobic amino acids (Kawasaki et al., 1998). By exploring the protein structures of FvCaMs, we gained a deeper understanding of the mechanism by which Ca$^{2+}$ binds to CaMs, which will undoubtedly contribute to future research.

Expression of CaM and CML Genes in Different Strawberry Tissues

Tissue specification of CaMs and CMLs is essential for protein interactions. In the present study, we tested the transcript abundance of the four FvCaM genes and four FvCML genes...
that are similar to CaMs in six different tissues (leaf, stem, root, bud, flower, and fruit) with the leaf examined in two stages (young and mature). As shown in Supplementary Figure S3, the eight genes were expressed in almost all of the tested tissues, yet some differences were still detected. For example, FvCaM2 showed a high expression level in all of the tissues, while FvCaM4 exhibited a steady low level of expression. In addition, FvCaM1 was expressed at much higher levels in young than in old leaves. Notably, these eight genes were steadily and highly expressed in the bud, flower and fruit (Supplementary Figure S3), supporting their potential roles in plant reproductive growth and development processes. Exploring the expression of FvCaM and FvCML genes in different tissues will facilitate further investigations of their interacting proteins and their functions in calcium signaling.

Expression Profile of FvCaMs and Several FvCMLs in Response to Multiple Treatments

To gain insights regarding the potential functions of FvCaMs and FvCMLs, we tested the transcription levels of the four FvCaM genes and the four most-related FvCML genes using RT-PCR following exposure to multiple treatments, including biotic and abiotic stresses as well as hormone treatments. Overall, FvCaMs and FvCMLs did not exhibit large changes in transcript abundance in response to the stress treatments; by contrast, some of them showed a distinct up-regulation in response to hormone treatment.

Strawberry PM was inoculated onto leaves as a biotic stress treatment, and drought, salt, and heat stress treatments were performed to simulate abiotic stress (Figure 5; Supplementary Figure S1). The FvCaM and FvCML genes exhibited insensitive expression patterns in response to multiple stimuli. However, some of these genes were clearly regulated. FvCaM1 and FvCML28 were strongly down-regulated (Figure 5), and the transcript abundance of FvCaM1 immediately decreased at 24 hpi, returning to nearly normal levels at 120 hpi. In contrast, the transcript abundance of FvCML28 decreased throughout the treatment period (Figure 5). In addition, FvCaM3 was slightly down-regulated (Figure 5). Despite the distinct down-regulation patterns of the three genes discussed above, the responses of FvCaMs and FvCMLs to abiotic stress were more ambiguous. Only FvCML7 showed a distinct change in expression at 72 hpt
in response to drought treatment, with a subsequent return to normal expression levels after 24 h of watering (Figure 5). In addition, \textit{FvCaM1} was slightly up-regulated in response to drought stress, even after the watering recovery period (Figure 5). For NaCl and the 42°C treatment, the expression patterns were more complex, and the expression levels fluctuated. It is worth mentioning that \textit{FvCML15} and \textit{FvCML24} were almost unresponsive to the three abiotic stress treatments (Figure 5), likely indicating that these two genes do not play a role in responses to abiotic stress.

The expression profiles in response to the hormone treatments revealed a distinct up- or down-regulation of the evaluated genes (Figure 5; Supplementary Figure S2). Among the eight chosen genes, \textit{FvCaM1} was remarkable because it was up-regulated throughout the entire treatment period in response to hormones, excluding Eth treatment, and it showed an especially strong up-regulation and reached peak levels of more than fivefold in response to SA treatment (Figure 5). Following ABA treatment, \textit{FvCML15} also displayed an increase in transcript abundance from 0.5 to 12 hpt, returning to normal levels at 24 hpt (Figure 5). In contrast, \textit{FvCML15} remained unexpressed during the whole treatment period (Figure 5). In addition to ABA treatment, several genes also responded to MeJA treatment. \textit{FvCaM1}, \textit{CaM4}, \textit{CML7}, and \textit{CML15} were positively regulated following MeJA treatment (Figure 5). Among them, \textit{FvCaM1}, \textit{CaM4}, and \textit{CML7} showed an immediate up-regulation, but only \textit{FvCaM4} exhibited an obvious peak at 8 hpt (Figure 5). In contrast, \textit{FvCML15} was only up-regulated at 12 hpt and peaked at values of more
FIGURE 5 | Expression of four CaM and four CML genes in response to stress and hormone treatments in strawberry (Fragaria vesca). Four stress treatments (powdery mildew, drought, NaCl and 42°C) (A) and four exogenous hormones (ABA, MeJA, Eth and SA) (B) were applied to the plants, and gene expression was measured by semi-quantitative reverse transcription PCR (RT-PCR). The Fv18S gene served as an internal control. The transcript abundance was measured and digitized using GeneSnap software and is indicated as the fold-change of the experimental treatments relative to the control samples visualized in a heat map. The color scale represents the log2 expression values, with red indicating increased transcript abundance and blue indicating decreased transcript abundance.

Subcellular Localization of FvCaMs and FvCMLs

The interactions of CaMs and CMLs with downstream proteins rely on the distributions and localizations in plant cells. To provide more cellular evidence, we cloned and isolated two FvCaM (FvCaM2 and FvCaM3) genes and three FvCML (FvCML7, FvCML15, and FvCML28) genes to assess their subcellular localization. GFP served as a control. The fusion proteins were transformed into prepared Arabidopsis mesophyll cell protoplasts and observed using an Olympus fluorescence microscope. Our results revealed that FvCaM2 and FvCaM3 specifically localized in the nucleus, while FvCML7 and 28 were distributed throughout the whole cell, which was similar to the localization pattern observed for the GFP control (Figure 6). Another CML gene, FvCML15, was only detected in the plasma membrane and cytosol, but no specific nuclear localization signal was detected. Intriguingly, as the spearhead in the calcium signaling pathway, FvCaM2 and FvCaM3 did not display a plasma membrane localization pattern but were restricted to the nucleus (Figure 6), which might be due to downstream interacting proteins that are present in the nucleus. By contrast, the three FvCMLs were localized on the plasma membrane and throughout the cytosol; FvCML7 and FvCML28 were also localized in the nucleus (Figure 6), indicating their multiple roles in calcium signaling. These findings for FvCaMs and FvCMLs will facilitate further functional analyses of their targeted proteins.

In the present study, multiple treatments (4°C, 42°C, H2O2, SA, MeJA, and Eth) were applied to Arabidopsis protoplasts carrying pFvCaMs-GFP or pFvCMLs-GFP to explore changes in the subcellular localization of the two CaMs (FvCaM2, 3)
and three CMLs (FvCML7, 15, 28). However, protoplasts treated with 42°C, H$_2$O$_2$ and SA were damaged and the fluorescence signal cannot be detected (data not shown). For the other three treatments (4°C, MeJA and Eth), the genes that showed a change in subcellular localization patterns are shown in Figure 7, while the genes that exhibited no changes in localization are not shown. Overall, FvCaMs and CMLs displayed a lower fluorescence intensity in response to the 4°C treatment, but the fluorescence intensity increased following treatment with hormones (MeJA and Eth). Under the 4°C treatment, a clear fluorescence signal for FvCaM3 was observed diffusely throughout the cytosol and nucleus, in contrast to the restricted localization to the nucleus under normal conditions (Figure 7C). In addition, following treatment with MeJA or Eth, FvCaM3 exhibited additional localization signal to the plasma membrane (Figures 7A, B). Only FvCaM3 showed clear changes in localization in response to the 4°C and Eth treatments; however, MeJA treatment resulted in changes in four genes (FvCaM2, CaM3, CML15, and CML28) (Figure 7A). The most intriguing one was FvCaM2, which exhibited a unique localization pattern. Compared with normal conditions, FvCaM2 displayed additional localization in the cytosol and, most likely, on chloroplast membranes (Figure 7A). Moreover, FvCML15 exhibited a distinct fluorescence signal in the nucleus, and the membrane localization signal of FvCML28 was lost (Figure 7A). Among the five tested genes, the localization of FvCaM3 changed in response to all of the evaluated treatments, while FvCML7 remained unchanged. Changes in the localization patterns of FvCaMs and CMLs will aid in elucidating their functions and interacting proteins in response to multiple treatments.

### Transient Expression Assays for FvCaM and FvCML Genes

In this study, we transiently over-expressed FvCaM and FvCML genes in N. benthamiana leaves via Agrobacterium-mediated transfection. We also applied treatments with 10 mM CaCl$_2$ and 10 mM EGTA as described previously (Reddy et al., 2011b) to provide the conditions of presence or absence of Ca$^{2+}$. As shown in Figure 8A, these five genes did not induce HR or any other visible phenotypes following their transient over-expression in N. benthamiana leaves. In addition, these results were not changed by either CaCl$_2$ or EGTA treatment. Because the tested FvCaMs and FvCMLs could not induce ROS burst, we further treated the infiltrated leaves with additional stimuli.

CaM and CML genes were found to participate in plant responses to biotic stress. Therefore, we conducted further experiments to evaluate the roles of FvCaMs and FvCMLs in the plant immune system. To avoid the systemic disease caused by pathogen inoculation, which might affect the control sites, we used an elicitor protein, INF1, which can induce the HR and cell death in N. benthamiana leaves, as previously reported (Kamoun et al., 1998). As shown in Figures 8B, C, the five FvCaMs and FvCMLs had no effect on the HR caused by INF1 (spot g), likely because these genes do not participate in pathways that are responsive to INF1. Intriguingly, we found that some of the spots that infiltrated by A. tumefaciens had HR phenotype (spot f, marked by red circles) (Figure 8C). In contrast, no HR was observed at the spots of over-expressed CaM/CMLs (spot e, infiltrated with GV3101 containing pFvCaM/CML-GFP and GV3101 alone, OD$_{600}$ = 0.5) (Figures 8B, C). Among the five genes (FvCaM2, CaM3, CML7, CML15, and CML28), the infiltrated leaves of FvCML15 did not show a HR response at spot f, while the other four genes displayed at least one transformed leaf with HR at spot f. In addition, the number of the infiltrated leaves that showed the HR phenotype at spot f differed among the four genes: FvCaM2 (3/9), FvCaM3 (3/9), FvCML7 (7/9), FvCML28 (2/9). These results indicated that FvCaM and FvCML contributed to the resistance of plants to A. tumefaciens and might participate in the immune response.

### DISCUSSION

#### Identification of FvCaM and FvCML Genes

Calmodulin, which functions as a Ca$^{2+}$ sensor, is conserved among eukaryotic cells (DeFalco et al., 2010). In addition to the
conserved CaMs, plants possess an extended family of CMLs. In the present study, we identified 4 FvCaM genes and 36 FvCML genes (Table 1). Due to a large number of genes encoding proteins that are similar to FvCaMs, we distinguished genes as FvCMLs based on sequence identities of more than 16%, as described for Arabidopsis (McCormack and Braam, 2003). Compared with the typical CaM genes (FvCaM1 and FvCaM2), FvCaM3 and FvCaM4 varied in gene length, especially FvCaM4, which was found to be much longer than typical CaM genes and which possessed three introns (Figure 1C). However, considering that FvCaM3 and FvCaM4 contained the typical EF-hand motif and clustered in the CaM group in the phylogenetic tree (Figure 1), we defined these two genes as FvCaM genes and considered them as CaM gene variants. Although it clustered with other CaM genes, FvCML28 was not defined as a CaM gene due to the absence of the typical EF-hand motif (Figure 1).

Bioinformatics Analysis of FvCaM and FvCML Genes Revealed Their Functional Conservation and Divergence

The conservation and divergence of gene structures leads to functional similarities or differences. Structural characteristics, such as the organization of motifs and exon–intron structures, provide insights concerning the evolution and function of FvCaMs and FvCMLs. All CaMs and CMLs are composed of EF-hands (McCormack et al., 2005). The number of EF-hands in CaMs and CMLs range from one to six (McCormack and Braam, 2003; Boonburapong and Buaboocha, 2007). However, only three EF-hands have been found in strawberry: two, three and four (Table 1). The EF-hand domain is conserved among FvCaMs (Figure 4C) but variable among FvCMLs (data not shown). Although FvCaM3 and FvCaM4 have different gene lengths and variable amino acid sequences, they still display conserved key sites, such as the EF-hand motif, and are considered to contain a typical EF-hand domain (Figure 4). The crystal structure of AtCaM7 has been described (Tidow et al., 2012). FvCaM1 has a sequence identity of 96.64% with AtCaM7, and we predicted the 3D structure of FvCaM1 protein using AtCaM7 as template (Figure 4B). These findings facilitated our exploration of the protein structure of FvCaM1 and the mechanism by which FvCaM1 binds to Ca$^{2+}$. The great similarity of the sequences and protein structure between AtCaM7 and FvCaMs indicate their similar functions.

In eukaryotic cells, CaM gene have rarely varied during their evolutionary history, while CMLs are very diverse (DeFalco et al., 2010). Based on the phylogenetic tree (Figure 2), CaMs from Arabidopsis, rice and strawberry clustered within a group and were distinct from CMLs. FvCMLs more often clustered with AtCMLs than with OsCMLs, suggesting that CMLs are conserved among eudicots compared with monocots. Intriguingly, subgroup 5 contained five AtCMLs and three FvCMLs but no OsCML (Figure 2), probably because this cluster is only present in eudicots. Synteny analysis between Arabidopsis and strawberry provided additional information regarding the evolution of FvCaMs and FvCMLs. Intriguingly, FvCaMs, which
are very conserved proteins evolutionarily, failed to be detected in synteny blocks with Arabidopsis. In contrast, five pairs of CMLs were recognized as syntenic gene pairs, and each pair of genes was found to be phylogenetically closely related (Figures 2 and 3). AtCML8 and AtCML11 possess two syntenic genes (FvCML15 and FvCML28) in the strawberry genome, indicating that FvCML15 and FvCML28 arose prior to the divergence of Arabidopsis and strawberry. These syntenic genes are likely to have the same or similar functions (Ghiurcuta and Moret, 2014).

The Expression Profiles and Subcellular Localizations of FvCaM and FvCML Genes Revealed Their Versatile Roles in Response to Multiple Stimuli

The roles of CaMs and CMLs during plant responses to stimuli and during plants growth and development have been widely examined (Reddy et al., 2011a). Based on previous microarray experiments, the expression patterns of individual members of the CaM/CML family have been shown to frequently differ spatially, temporally, and in magnitude in response to stimuli, suggesting specificity in their roles in signal transduction (Perochon et al., 2011). Our semi-quantitative RT-PCR data revealed an abundance of FvCaM and FvCML gene transcripts in response to multiple treatments and a spacial expression profile of tissues. Furthermore, stimuli can induce both spatial and temporal fluctuations in Ca^{2+} concentrations. As crucial mediators of calcium signaling, CaMs and CMLs respond to Ca^{2+} by interacting with numerous CBPs, which participate in the regulation of transcription, metabolism, ion transport, cytoskeleton-associated functions, protein phosphorylation, and phospholipid metabolism (Yang and Poovaiah, 2003; Bouche et al., 2005; Reddy et al., 2011a). These interactions rely on the specific locations in plant cells. As previously reported, the protoplast transient expression system is a powerful and versatile cell system for investigating gene expression in response to internal and external signals and for efficient and penetrating analysis of the underlying molecular mechanisms (Sheen, 2001; Yoo et al., 2007; Wu et al., 2014). A series of research used the Arabidopsis mesophyll protoplast transient expression system to study genes functions and signaling transduction (Kim and Somers, 2010; Zhang et al., 2015). Consequently, we used...
Our data showed that Ca
2+
relies on ABA. CaMs and CMLs are recognized as important positive regulators of ABA-mediated signaling and responses to pathogens, such as infection by A. tumefaciens. For example, FvCaM1 (Zhang et al., 2005), FvCML28 (Chiasson et al., 2006), and FvCML7 showed a decreased transcript abundance following inoculation with PM (Figure 5). The expression patterns are similar to those of two resistance-related CaMs, CaCaM1 (Zhang et al., 2006) and NiCaM13 (Takabatake et al., 2007), which indicated that these three genes might be involved in immune pathways. Notably, the functions of some CaMs are associated with Ca
2+
signaling and responses to SA, a hormone that is closely related to resistance. Through this pathway, CaMs were found to bind TFs (Du et al., 2009; Galon et al., 2010). Similarly, FvCaM1 was immediately and highly up-regulated in response to SA treatment (Figure 5). In summary, FvCaM1 is most likely to participate in immune signaling pathways (Supplementary Figure S4). In addition to FvCaM1, FvCaM4, and FvCML15 were also positively regulated by SA treatment (Figure 5), and might also participate in the immune system. As discussed above, FvCaMs and CMLs function indirectly by interacting with proteins in immune signaling pathways, and additional proteins remain to be identified to fully understand the roles of CaMs and CMLs in the immune system.

In addition to biotic stress, CaMs and CMLs also participate in responses to abiotic stress, such as drought (Kaplan et al., 2006), salt (Xu et al., 2011), and heat (Zhang et al., 2009), as well as hormone treatments. TFs, such as bZIP and MYB, are considered to be the major CaM binding proteins in such pathways (Jakoby et al., 2002; Abe et al., 2003). The tolerance of plants to drought relies on ABA. CaMs and CMLs are recognized as important Ca
2+
sensors in the ABA-mediated pathway (Kaplan et al., 2006). Our data showed that FvCML7 had a distinct transcriptional response to drought stress, while FvCaM1 had a slight response (Figure 5). This result is consistent with the expression pattern observed in response to ABA treatment, which resulted in the positive regulation of FvCaM1 and FvCML7 (Figure 5). Thus, these two genes tended to participate in the drought stress response (Supplementary Figure S4). In addition, a series of TFs are involved in the drought stress response, such as ABF/AREB (Fujita et al., 2005; Yoshida et al., 2010), MYB (Abe et al., 2003), and NAC (Rizhsky et al., 2004), and the binding of these TFs to CaMs occurs in the nucleus. Nevertheless, additional studies are needed to identify the specific TFs bound by FvCaMs and their corresponding signaling pathways. Compared with drought stress, a more subtle response was observed in strawberry following exposure to other abiotic stresses. In response to heat stress, the CaM3 knockout Arabidopsis mutant displays impaired thermotolerance, whereas overexpression of AtCaM3 increases thermotolerance (Zhang et al., 2009). The phylogenetically closely related AtCaM3 gene in strawberry is FvCaM2, which also showed an increased transcript abundance in response to heat stress (Figure 5), indicating that it responds to heat stress similarly to AtCaM3. Additional findings revealed that CaMs participate in heat stress responses by interacting with HSFs (Liu et al., 2007), providing evidence that FvCaM2 is restricted to the nucleus (Figure 6). Intriguingly, FvCaM2 only responded to the heat stress treatment and was insensitive to other stress and hormone treatments (Figure 5). These results emphasize the significance of FvCaM2 responses to heat stress and the strong possibility that FvCaM2 participates in heat response pathways (Supplementary Figure S4). In terms of NaCl stress, the responses of CaMs and CMLs have been published (Yoo et al., 2005; Galon et al., 2010). A large number of CaM-binding proteins induced by salt stress have also been identified in the nucleus. For example, the CAMTA TF (Galon et al., 2010) and CaMBP25 are nuclear CaM-binding proteins that are induced in response to multiple abiotic stresses (Perruc et al., 2004). In our study, FvCaMs and FvCMLs were insensitive to NaCl treatment (Figure 5), potentially due to the response to salt stress reflected in the activation of CaMs and CMLs proteins but not in their transcription levels. Notably, for the treatments performed in Arabidopsis protoplasts herein, the most attractive gene was FvCaM3, which showed additional plasma membrane localization in response to the MeJA, Eth and 4°C treatments (Figure 7). These results might be due to its special function in Ca
2+
signal transduction in response to treatment. However, more additional functional investigations are needed to confirm this hypothesis. One of the most important things for future research should be the identification of the physiologically relevant protein targets. Recent developments in protein interaction analysis, such as proteome chips, tandem affinity purification (TAP) and mass spectrometry could help to identify the downstream targets of CaMs and CMLs. And to further investigate the spatial and temporal distribution and the formation of protein complex in vivo, some cell biological approaches (e.g., bimolecular fluorescence complementation, fluorescence resonance energy transfer and other emerging technologies) could be used.

**Transient Expression Assays Suggested That Over-Expression of Four FvCaM/CML Genes Increased Resistance to Agrobacterium tumefaciens**

Agrobacterium-mediated transient expression methods by leaf infiltration have been well-developed in Nicotiana species (Sheuldke et al., 2007). As an efficient and versatile way, transient expression of exogenous genes in N. benthamiana leaves can provide preview of genes functions, especially for the
HR phenotype (Goodin et al., 2008; Hao et al., 2014; Ouyang et al., 2014). A series of transient assays of CaM genes have been previously reported. Transient over-expression of Capsicum annuum calmodulin 1 (CaCaM1) activated the ROS burst and NO generation, and it induced HR-like cell death in pepper leaves (Choi et al., 2009). Silencing of Solanum lycopersicum SICaM2 and SICaM6 resulted in reduced resistance to Tobacco rattle virus and the oomycete pathogen, Pythium aphanidermatum, via VIGS (Zhao et al., 2013). Accordingly, we transiently expressed five FvCaM and FvCML genes in N. benthamiana leaves and exposed them to both the presence (Ca2+ treatment) and absence (EGTA treatment) of Ca2+. However, no significant HR phenotype was found (Figure 8A). Furthermore, we used an elicitor protein INF1 to evaluate whether over-expression of FvCaM/CML could increase or reduce H2O2 accumulation or the HR phenotype, yet no effects were observed (Figures 8B,C). However, we found that some sites with infiltrated A. tumefaciens containing pBl221 plasmid accumulated H2O2 and showed a slight HR phenotype (Figure 8C). In contrast, none of the sites that were infiltrated with A. tumefaciens containing pFvCaM/CML-GFP displayed HR phenotype (Figure 8B). As previously reported, plant resistance to Agrobacterium relies on the perception of PAMP, EF-Tu (Cyril et al., 2006). However, N. benthamiana, a plant that is unable to perceive EF-Tu, is highly sensitive to Agrobacterium-mediated transformation (Wroblewski et al., 2005). As previously supported, the HR and cell death induced by A. tumefaciens have been found in N. benthamiana and other species (Choi et al., 2009; Hao et al., 2014). Taken together, our results suggested that at least four FvCaM/CML genes (FvCaM2, CaM3, CML7, and CML28) could provide beneficial effects for plant resistance to A. tumefaciens. Because FvCaMs and FvCMLs were not responsible for INF1 and did not function as TFs or PRs (pathogenesis-related proteins), the observed resistance to A. tumefaciens might not be a result of their specific functions and might not be associated with a specific pathway. We postulate that the increased resistance of N. benthamiana leaves might be attributable to the following three factors: (i) Ca2+ signaling could be induced by stimuli (in this study, by our treatments); (ii) FvCaM/CML play roles upstream of Ca2+ signal transduction; (iii) over-expression of FvCaM/CML enhanced the perception and conduction of Ca2+ signaling, which improved the adaptation of the plants to the environments. Stable transformation experiments, including Arabidopsis mutants and transgenic strawberry plants, should be conducted to validate the functions of the FvCaM/CML genes.

CONCLUSION

Calmodulins and CMLs act as bridge to connect Ca2+ signal and downstream CBPs and play vital roles in plants response to stimuli (Zhang and Lu, 2003; Lecourieux et al., 2006). During the past years, significant progress in our knowledge of the CaMs/CMLs in plants has been achieved (Perochon et al., 2011) in (i) identifying unique functions among members of this gene family, (ii) demonstrating the protein interactions between CaMs/CMLs and the targeted proteins, and (iii) describing the dynamic network that regulated by Ca2+. In this study, we identified 4 CaM and 36 CML genes in woodland strawberry (F. vesca). FvCaMs are conserved among eudicots, which compared with monocots. FvCMLs can be divided into four subgroups on the basis of phylogenetic relationship and gene structure. The transcript abundance of the tested FvCaMs and FvCMLs showed a distinct spacial and temporal regulation in different tissues and in response to multiple treatments. The comprehensive analysis combining the bioinformatics data and the experimental evidence indicated a series potential functions of FvCaMs and FvCMLs in biological process. The most intriguing genes and processes should be FvCaM1 in biotic stress, FvCaM2 in heat stress and FvCaM1/7 in drought stress. However, a major challenge that remains is to further verify these predictions, determine their specificity, and test their biological significance. Overall, our study laid a foundation for further exploring the biological roles of CaMs and calmodulin-like proteins in strawberry plants.

AUTHOR CONTRIBUTIONS

KZ, JF, and ZZ conceived and designed the research. KZ carried out bioinformatics analyses. KZ, DY, and WW performed all treatments and RT-PCR tests. KZ and YH performed subcellular localization experiments. KZ performed transient expression assays in N. benthamiana leaves. KZ and JF analyzed and interpreted the data. KZ drafted the manuscript. ZZ and JF contributed with consultation. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01820/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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