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Differential expressions of citrus CAMTAs during fruit development and responses to abiotic stresses

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Abstract

Calmodulin-binding transcription activators (CAMTAs) play important roles in plant growth, developmental processes, and responses to abiotic and biotic factors. Recently, five CAMTA members were identified in *Citrus sinensis*, however, very little is known about the molecular regulation of these CAMTAs in citrus during fruit development and under abiotic stresses. In this study, the different expression profiles of *CsCAMTA* genes were found in different tissues and different fruit developmental stages. The *CsCAMTA* genes also displayed distinct expression patterns after heat, cold, salt, and drought stresses. Furthermore, the expressions of *CsCAMTA* genes were significantly induced by treatments with salicylic acid, methyl jasmonate, or abscisic acid. The green fluorescent protein gene fused with *CsCAMTA* was specifically expressed in the nucleus of *Nicotiana benthamiana* cells. Additionally, CsCAMTA proteins can activate or suppress DNA transcription in yeast. These findings provide helpful information for further studies of stress signals in citrus.

Additional key words: abscisic acid, *Citrus sinensis*, cold, drought, heat, jasmonic acid, *Nicotiana benthamiana*, salicylic acid, salinity, yeast.

Introduction

Calcium is a ubiquitous cellular regulator involved in a wide range of physiological processes and in response to both biotic (pathogens and insect feeding) and abiotic stresses (low temperature, salt, and drought) (Liu *et al*. 2015, Gilroy *et al*. 2016, Cao *et al*. 2017, Ribaudo *et al*. 2017, Wei *et al*. 2017). In plant cells, calcium is perceived by the EF-hand domain containing protein families, including calmodulin (CaM)/CaM-like proteins, calcium dependent protein kinase (CDPK), and calcineurin B-like proteins (CBL; Tsuda and Somssich 2015, Zhou *et al*. 2015, Ranty *et al*. 2016, Mohanta *et al*. 2017, Xiao *et al*. 2017). Among these proteins, CaM is a well characterized Ca^{2+}-binding protein, and it functions as a modulator in metabolism, ion transport, transcriptional regulation, protein phosphorylation, and other critical functions. Calmodulin modulates the activity of a certain CaM-binding proteins, which after binding to Ca^{2+}, generate physiological responses to various stimuli (Leng *et al*. 2015). To date, over 90 transcription factors have been identified as CaM-binding proteins including calmodulin-binding transcription activators (CAMTAs, also known as signal regulators) (Fromm and Finkler 2015, Yang *et al*. 2015).

The CAMTAs are well characterized by a CG-1 DNA-binding domain that binds to CGCG or CGTG core motifs at the N terminus and are found in all multicellular eukaryotes (Bouché *et al*. 2002). Apart from the CG-1 DNA-binding domain, the structural analysis also shows that CAMTA proteins contain other conserved regions arranged in the same colinear order such as a TIG domain (involved in a nonspecific contact with DNA and in the dimerization of transcription factors), two ankyrin (ANK)

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Abbreviations: ABA - abscisic acid; CaM - calmodulin (Ca^{2+}-binding protein); CAMTA - calmodulin-binding transcription activator; DAFB - days after full blossom; ET - ethylene; GFP - green fluorescent protein; MeJA - methyl jasmonate; NLS - nuclear localization signal; RT-qPCR - reverse transcription quantitative PCR; SA - salicylic acid.

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repeats (implicated in a protein-protein interaction), a variable number of IQ motifs (associated with binding of CaM and CaM-like proteins), and a CaMB domain in the C-terminus (Finkler *et al*. 2007). Recently, multiple CAMTA family proteins were characterized in several species including *Arbabidopsis thaliana*, *Solanum lycopersicum*, *Vitis vinifera*, *Fragaria* × *ananassa*, *Brassica napus*, *Zea mays*, *Brassica campestris*, *Medicago truncatula*, *Glycine max*, and *Populus trichocarpa* (Yang and Poovaiah *et al*. 2002, Yang *et al*. 2012, Shangguan *et al*. 2014, Wang *et al*. 2015, Hu *et al*. 2015, Leng *et al*. 2015, Yang *et al*. 2015, Yue *et al*. 2015, Rahman *et al*. 2016a, Wei *et al*. 2017).

The CAMTAs differentially respond to low temperature, wounding, pathogen infection, drought, salinity, and treatments with phyothormones salicylic acid (SA), methyl jasmonate (MeJA), ethylene (ET), and abscisic acid (ABA) (Shen *et al*. 2015). In *Arabidopsis*, an AtSR1 DNA-binding domain can bind the Enhanced disease susceptibility 1 (EDS1; an important positive regulator of SA biosynthesis) promoter fragment in an ACGCGT-dependent way to repress it. The expression of EDS1 and SA content are elevated in *Atsr1* mutants (Du *et al*. 2009). Furthermore, *AtSR1/CAMTA3* loss-of-function mutants increase resistance to a fungal pathogen *Pseudomonas syringae* and a bacterial pathogen *Botrytis cinerea* (Galon *et al*. 2008) but decrease resistance against insect herbivores. Recently, Kim *et al*. (2017) found that AtCAMTA3 mediated repression of SA pathway involves the action of an N-terminal repression module binding to the IQ and CaMB domains in response to low temperature. Additionally, tomato *CAMTAs* were reported to be differentially expressed during fruit development and ripening suggesting their role in these processes (Yang *et al*. 2012).

Citrus is a perennial tree grown worldwide for the production of fresh fruits and juice among other products (Tan and Swain 2007). A recent genome-wide bioinformatics identified five *CAMTA* genes in the *Citrus sinensis* genome (Rahman *et al*. 2016b). However, our understanding of CAMTA functions in citrus remains elusive. With the aim to elucidate the biological functions of CAMTAs in citrus, we investigated the expressions of *CsCAMTAs* in various tissues and different stages of fruit development. Furthermore, we studied responses of CsCAMTAs to various abiotic stresses (heat, cold, salt, and drought) and phytohormones (SA, MeJA, and ABA). Finally, the subcellular location and transcriptional activation of five CAMTA members were investigated.

Materials and methods

Domain analyses of CsCAMTA proteins: Protein sequences of CsCAMTAs were downloaded from the *Phytozome* (http://www.phytozome.net) and the *Citrus Genome* databases (https://www.citrusgenomedb.org). Multiple sequence alignments of the full-length CsCAMTA proteins were conducted using the *Clustal X* program and were viewed by *GeneDoc* (http://www.nrbsc.org/gfx/ genedoc/). Domain analyses of CsCAMTA proteins were predicted using the *Pfam* (http://pfam.sanger.ac.uk/) and the *Motif Scan* (http://myhits.isb-sib.ch/cgi-bin/motif_scan) programs.

Plants and treatments: *Citrus sinensis* Osbeck cv. Gannanzao was grafted on trifoliate orange and grown in plastic pots containing a mixture of sand, soil, and plant ash (1:1:1, v/v/v) under fluorescent tubes (an irradiance of 200 μmol m^2 s^{-1}), a 14-h photoperiod, a temperature of at 22 - 24 °C, and a relative humidity of 65 %. For tissue and organ-specific expressions of *CsCAMTA* genes, mature leaves, stems, and lateral roots were collected from seedlings approximately two months after grafting. Flowers at full bloom and fruits at 110, 140, 160, 180, and 200 d after full blossom (DAFB) were collected from 4-year-old citrus trees grown in a glasshouse. Tissue material was frozen in liquid nitrogen and stored at -80 °C until use.

The citrus seedlings two-month-old after grafting were subjected to various treatments. For drought and salinity stresses, seedlings were transferred from hydroponic cultivation to a layered filter for rapid dehydration or kept in a liquid medium with 200 mM NaCl, respectively. For cold or heat treatment, culture tubes were transferred to incubators maintained at 4 ± 1 °C or 42 ± 1 °C for 8, 24, and 48 h, respectively. Citrus seedlings kept at 28 ± 1 °C were used as a negative control. For hormone treatments, leaves were sprayed with 100 mM SA, 100 μM MeJA, or 100 μM ABA solutions containing 0.1 % (v/v) ethanol and 0.02 % (v/v) *Tween-20* and kept under the normal growth conditions. Plants that were sprayed with a solution containing only 0.1 % ethanol and 0.02 % *Tween-20* were used as a negative control. All leaf samples harvested at indicated times were stored at -80 °C until future use.

Extraction of RNA and cloning CsCAMTA genes: Total RNA was extracted from treated and control samples using a *TRIZOL* reagent (*Invitrogen*, Shanghai, China) and treated with *RNase-free DNase I* (*TaKaRa*, Dalian, China). Approximately 1 μg of the total RNA was used to synthesize first-strand cDNA using a *Superscript III RT Reagent* kit (*Invitrogen*, Shanghai, China) according to the manufacturer's instructions. The full open reading frame of *CsCAMTA* genes was amplified based on the predicted sequence using special primers listed in Table 1 Suppl. The amplified cDNA fragments were cloned and sequenced.

Reverse transcription quantitative PCR (RT-qPCR)

was used to test the expression patterns of *CsCAMTA*s. The RT-PCR reactions were conducted in a total volume of 25 mm^3 containing 1 μg of cDNA, 12.5 mm^3 of *SYBR Green Master Mix* (*TaKaRa*) and specific primers (3 pM). The RT-qPCR was performed on a 96-well plate (*BIO-RAD CFX96 q-PCR* system, Hercules, CA, USA) under following conditions: an initial denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 57 °C for 60 s. Quantification analysis was conducted using by the *CFX Manager* software. Three independent biological replicates were used for analyses, and the relative expressions were calculated using the $2^{-\triangle\triangle CT}$ method. Primers used are listed in Table 1 Suppl. Citrus *CsActin* (Gene ID: 102577980) was used as an internal control with primers CsActin-F and CsActin-R (Xie *et al*. 2014), and the relative expressions of the target genes were shown as a fold of the expression of the actin gene (Table 1 Suppl.).

Subcellular localization: The coding sequences of *CsCAMTA* genes were amplified by PCR with specific primers (Table 1 Suppl.). The PCR products were digested with *Xba*I and *Sam*I restriction enzymes and inserted into the corresponding *Xba*I/*Sam*I site of a vector pFGC-Egfp to obtain pFGC-Egfp:CsCAMTA1-5. The sequence-verified constructions and pFGC-Egfp were transformed into *Agrobacterium tumefaciens* GV3101. Four-week-old *Nicotiana benthamiana* Domin plants were inoculated with the transformed *Agrobacterium* cultures and then grown at 25 °C for 48 h. Fluorescence signals were excited at 488 nm and detected using a 500 - 530 nm emission filter performed with a confocal laser scanning microscope (*Zeiss LSM 510 META*, Oberkochen, Germany).

Transcription activation assay in yeast: The full open reading frame sequence of *CsCAMTA* genes was amplified using the primers listed in Table 1 Suppl. The amplicons were cut with restriction enzymes and inserted into the appropriate site of a pBD-GAL4Cam vector, resulting in pBD-CsCAMTAs. The recombinant plasmids and the pBD empty vector (negative control) were transformed into yeast strain AH109 and cultivated on SD/-Trp and SD/-Trp/-His media followed by supplementation with β-D-galactopyranoside (X-α-galactose) at 28 °C for 3 d.

Statistical analysis: The *SAS* statistical software package *v. 9.4* was used for statistical analysis. Experiments were repeated three times, and three replicates were included in each experiment. Data obtained from the three independent experiments were analyzed by the Dunnett *t*-tests, taking $P < 0.05$ as a significant difference.

Results

The *Motif Scan* program was used to complete domain analyses of CsCAMTA proteins. As predicted, all CsCAMTA proteins contained one CG-1 DNA bind domain in the N-terminus, two ankyrin repeats, two or three IQ motifs, and one Ca^{2+} dependent calmodulin binding domain (CaM-binding domain) in the C-terminus (Fig. 1 Suppl.). Except for CsCAMTA5, all other CsCAMTA proteins contained one TIG domain. Additionally, one or two nuclear localization signals (NLS) domains were identified in all CsCAMTA proteins, consistent with their function in the nucleus (Wang *et al*. 2015). Analyses of the amino acid sequences indicate that all five CsCAMTAs were genuine homologs belonging to the CAMTA transcription factor family.

To determine the physiological functions of the different members of citrus *CAMTA* genes, we analyzed their tissue-specific expression patterns in different tissues, including roots, stem, leaves, flowers, and fruits by RT-qPCR. The results show that the five *CsCAMTA* genes were detectable in all the tested tissues but exhibited different expression patterns (Fig. 1). The *CsCAMTA1* and *CsCAMTA4* reached high expressions in leaves and

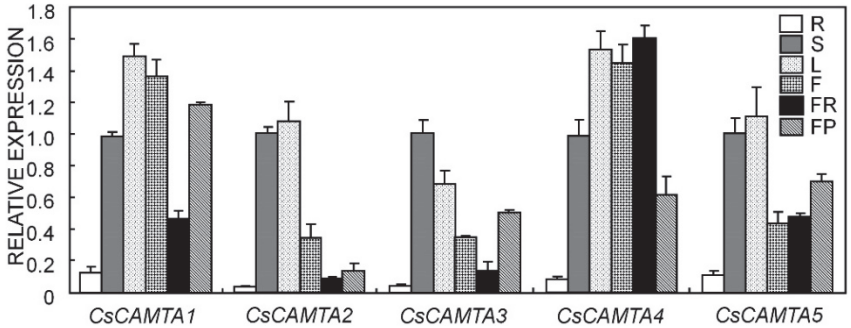

Fig. 1. Expression patterns of calmodulin-binding transcription activator (*CAMTA*) genes in roots (R), stems (S), leaves (L), flowers (F), fruit pulp (FR), and fruit peel (FP) of citrus plants. Root, stem, and leaf samples were collected from spring shoots (about two months after grafting). Flowers were collected at full bloom and fruits at 140 days after full blossom (DAFB) of 4-year-old trees. Relative expression to the control actin gene (*CsActin*). Means ± SDs, $n = 3$.

flowers but low in roots. The expression of *CsCAMTA3* in stems was higher than in other tissues. However, *CsCAMTA2* showed low expressions in stems, flowers, fruit peel, and fruit pulp. The *CsCAMTA5* was highly expressed in stems and leaves, but the expressions were relatively low in other organs, particularly in roots. Generally, all the *CsCAMTA* genes had relatively high expressions in stems and leaves and low in roots. The results suggest that *CsCAMTA* genes played various roles in different tissues.

To study the potential role of *CsCAMTA* genes in fruit development and ripening, we evaluated their expression patterns during five different developmental and ripening stages. Four *CsCAMTA* genes (*CsCAMTA1*, *2*, *4*, and *5*) in peel and all *CsCAMTA* genes in pulp were down-regulated at 200 DAFB compared with their expressions at 110 DAFB. Notably, *CsCAMTA3* expression in peel was constant in all the five tested stages (Fig. 2). The *CsCAMTA1* and *CsCAMTA5* expressions in peel increased from 110 to 160 DAFB and then decreased from 180 to 200 DAFB, whereas the expression of *CsCAMTA2* in peel firstly decreased but then increased at 160 DAFB and thereafter decreased gradually (Fig. 2). The expressions of *CsCAMTA2*, *3*, *4*, and *5* in pulp were relative high at 110 and 140 DAFB but decreased from 160 to 200 DAFB. The expression of *CsCAMTA1* from 140 to 180 DAFB in the pulp was significantly lower than in peel as well as those of *CsCAMTA3* from 160 to 200 DAFB and *CsCAMTA4* at 140 and 160 DAFB (data not shown). In general, the expressions of *CsCAMTAs* were lower at the later stages (160 to 200 DAFB) than in the early stages of fruit development and ripening in pulp, whereas expressions of four *CsCAMTA* genes were down-regulated at 180 and 200 DAFB in peel compared with 110 DAFB.

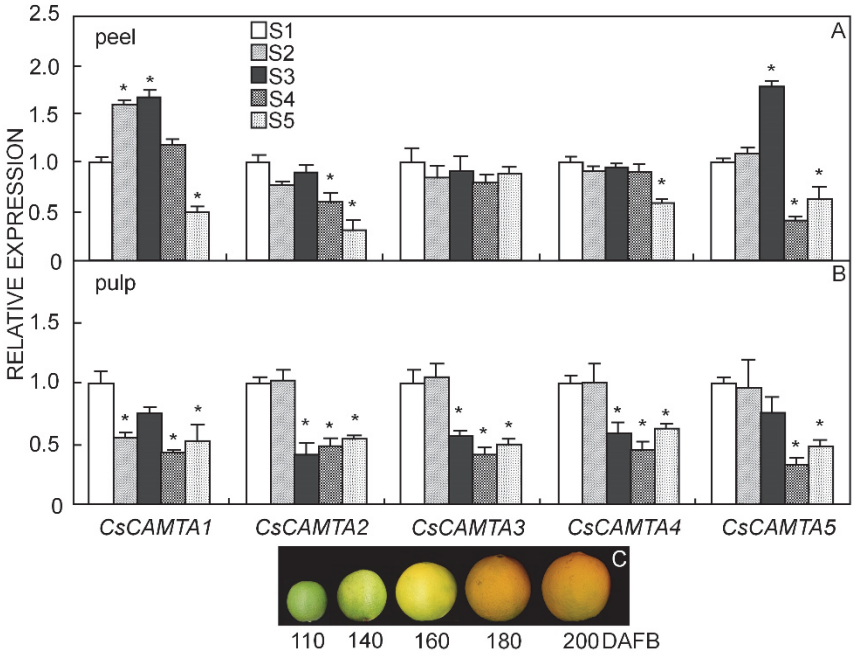

Fig. 2. Expression patterns of calmodulin-binding transcription activator (*CAMTA*) genes in fruit peel (*A*) and pulp (*B*) during different fruit development and ripening stages. *C* - Samples used in expression assay of *CAMTA* genes. S1 - 110 days after full blossom (DAFB), S2 - 140 DAFB, S3 - 160 DAFB, S4 - 180 DAFB, and S5 - 200 DAFB. A relative expression to the endogenous control *CsActin*. Means ± SDs, $n = 3$; * - significant differences between treatments at $P \leq 0.05$.

Drought, salinity and extreme temperature are the primary environmental stresses affecting plant growth/ development and ultimately crop yield. To investigate the molecular functions of *CsCAMTA* genes in response to abiotic stress, we analyzed the expressions of the five *CsCAMTA* genes in leaves of citrus seedlings under different stresses including heat (42 °C), cold (4 °C), drought (placing on a lab bench without water supply) and salt (drenching with 200 mM NaCl).

We first analyzed the expressions of the *CsCAMTA* genes in leaves of citrus seedlings after heat and cold treatments. In the heat treatment group, the expressions of four of the *CsCAMTA* genes (*CsCAMTA1*, *2*, *4*, and *5*) increased and displayed the highest expression (1.9- to 3.4-fold higher than in the control) at 24 h after heat treatment (Fig. 3). However, the expression level of *CsCAMTA3* did not show significant changes under the heat stress conditions. In the cold treatment assay, the expressions of all the five *CsCAMTA* genes was up-regulated and increased at 24 h, ranging from a 1.8- to 4.1-fold increase compared with control plants. Notably, *CsCAMTA2* was strongly induced at all time-points following treatment.

In response to drought stress, the expressions of the five

CsCAMTA genes were significantly affected. The expressions of three of the *CsCAMTA* genes (*CsCAMTA3*, *4*, and *5*) increased rapidly after drought treatment, resulting in 3.3- to 6.5-fold increase compared with the control. By contrast, *CsCAMTA1* and *CsCAMTA2* were down-regulated at all the tested time points (Fig. 4), the expression of *CsCAMTA1* was down-regulated significantly by >7-fold at 24 h after drought treatment. In the salt treatment group, three of the *CsCAMTA* genes (*CsCAMTA1*, *3*, and *5*) were affected at 24 h after NaCl treatment (Fig. 4). The expression of *CsCAMTA1* gradually decreased and peaked at 24 h after salt treatment,

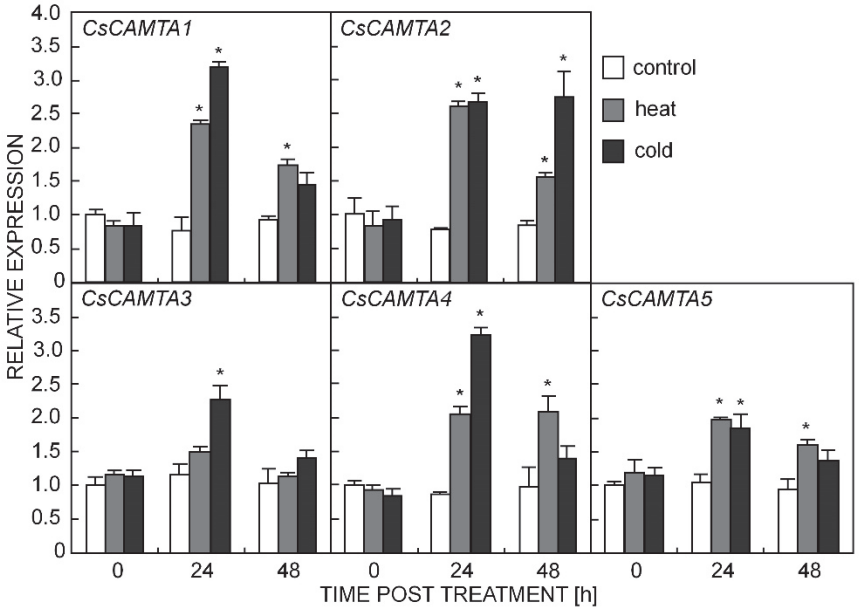

Fig. 3. Expression patterns of calmodulin-binding transcription activator (*CsCAMTA*) genes in response to cold and heat stresses. Cold and heat stresses were applied by placing two-month-old citrus seedlings to a temperature of 4 and 42 °C, respectively, for 0, 24, and 48 h. Controls were grown at 28 °C. Leaf samples were collected at the indicated time points for reverse transcription quantitative PCR analyses. Means ± SDs, $n = 3$; * - significant differences between treatments at $P \leq 0.05$.

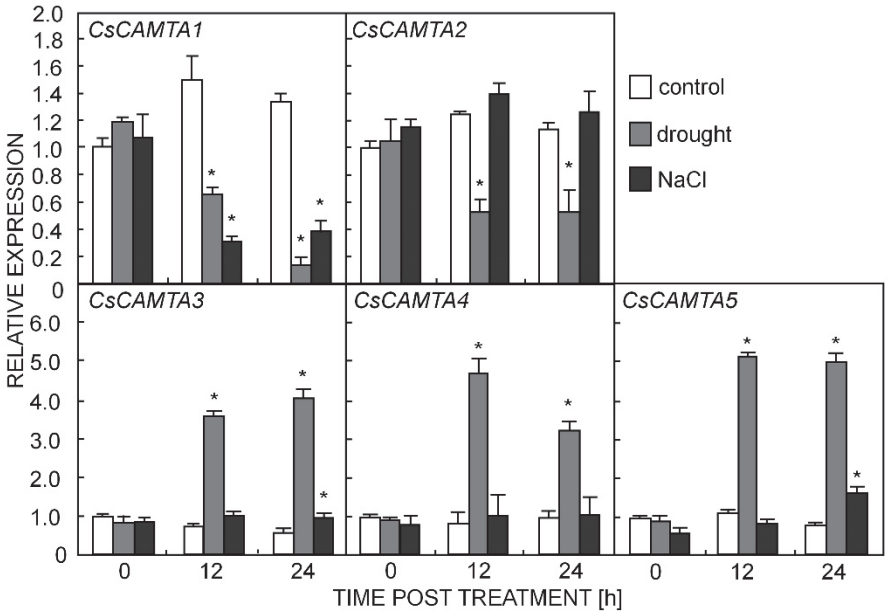

Fig. 4. Expression patterns of calmodulin-binding transcription activator (*CsCAMTA*) genes in citrus leaves after treatment with drought and salt stresses imposed to two-month-old citrus seedlings. Leaves were placed among three layers of filter paper for a fast dehydration or seedling roots were immersed in a 200 mM NaCl solution for 0, 12, and 24 h. Citrus leaves were collected at the indicated time points for reverse transcription quantitative PCR analyses. Means ± SDs, $n = 3$; * - significant differences between treatments at $P \leq 0.05$.

resulting in a 3.5-fold decrease compared with the control, whereas the expressions of *CsCAMTA3* and *CsCAMTA5* decreased significantly only at 24 h after treatment. The other two *CsCAMTA* genes showed no apparent changes in expressions under the salt stress conditions.

To examine the effects of SA, JA, and ABA on CsCAMTA expressions, we analyzed the expressions of these genes after the plants were sprayed with 100 mM SA, 100 μM MeJA, or 100 μM ABA (Fig. 5). The JA significantly increased the expressions of *CsCAMTA1* and *5* at 24 and 48 h after spraying, leading to a 1.6- to 2.2-fold increase in expressions compared with the control. However, no significant change occurred in the expressions of the other three *CsCAMTA* genes ($P < 0.05$). The expressions of *CsCAMTA1*, *4*, and *5* were induced by SA treatment with an increase of 1.6-, 4.5-, and 1.6-fold at 24 h after treatment, respectively. Among these three genes, the expression of *CsCAMTA4* exhibited a continuous increase and showed a significant up-regulation by 10.1-fold at 48 h after SA treatment. Furthermore, for all the *CsCAMTA* genes, the expressions increased from 1.5- to 2.8-fold at 24 and 48 h in ABA-treated plants (Fig. 5). The *CsCAMTA* genes responded to phytohormones by differential expression patterns, which might indicate biological functions in relation with stress hormone-mediated signalling.

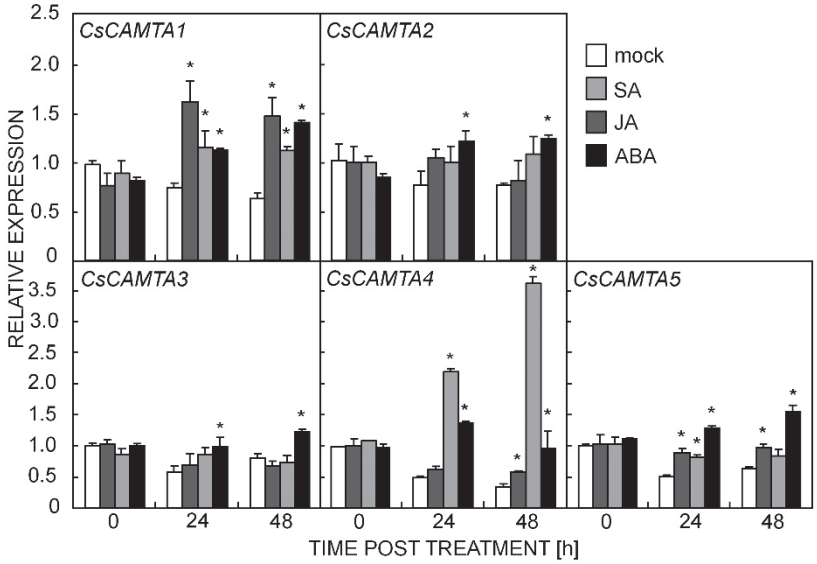

Fig. 5. Expression of calmodulin-binding transcription activator (*CsCAMTA*) genes in response to application of phytohormones. The leaves of two-month-old citrus seedlings were sprayed with 1 mM SA, 100 μM MeJA, 100 μM ABA, or an equal volume of solvent as a control. The expressions of target genes were analyzed by reverse transcription quantitative PCR at the indicated times after treatment. Means ± SDs, $n = 3$; * - significant differences between treatments at $P \leq 0.05$.

To investigate the subcellular localization of CsCAMTA proteins, the coding sequences of *CsCAMTA* genes were amplified and fused to the N-terminal of green fluorescent protein (*GFP*) under control of the CaMV 35S promoter, and then transiently expressed in *N. benthamiana* leaves. Subcellular localization of GFP in *N. benthamiana* leaves was examined by confocal microscopy. The results show that CsCAMTA:GFPs were exclusively localized to the nucleus, whereas the GFP alone was detected in both the nucleus and the cytoplasm (Fig. 6). These results demonstrate that CsCAMTA proteins were nuclear-targeted proteins.

Most transcription factors, such as *Oryza sativa OsERF3*, act as activators that positively regulate the transcriptions of their target genes (Lu *et al*. 2011). The five *CsCAMTA* genes were transformed into yeast cells. As shown in Fig. 7, yeast cells, harboring plasmids pBD-CsCAMTA and a pBD empty vector (as a negative control) grew normally on an SD/-Trp medium. However, the pBD-CsCAMTAs-containing yeast cells grew well also on an SD/-His/-Trp medium and produced a blue pigment after supplementation with x-α-galactose, excluding transformants containing the pBD empty vector (Fig. 7). These results suggest that CsCAMTA proteins functioned as transcriptional activators.

Discussion

The calmodulin-binding transcription activator is a universal family of multifunctional transcription factors, which has been identified in various eukaryote species. Comprehensive gene expression analysis of *CAMTA*

family genes revealed that *CAMTAs* have distinct expression patterns in various tissues of several plant species (Yang and Poovaiah 2002, Yang *et al*. 2012, 2013, Wang *et al*. 2015, Leng *et al*. 2015, Yue *et al*. 2015, Rahman *et al*. 2016a). Some members of the *CAMTA* family exhibit organ-specific expression patterns or are not expressed in some tissues, *e.g*., *AtCAMTA1* is specifically expressed in pollen (Mitsuda *et al*. 2003). Expressions of *SlSR3L* and *SlSR4* are only detected in tomato fruit (Yang *et al*. 2012), whereas *BnCAMTA3A2, A3C2* are nearly undetectable in all tested tissues of *Brassica napus* (Rahman *et al*. 2016a). By contrast, some members of *CAMTAs* have high expressions in special organs such as *FaCAMTA3* and *FaCAMTA4* in young berries of strawberry (Leng *et al*. 2015). The expression patterns of *CAMTA* genes vary greatly among different plant species. In this study, the *CsCAMTA* genes were expressed in all tested tissues, but they exhibited differential expression patterns (Fig. 1) suggesting that they play different roles in different tissues.

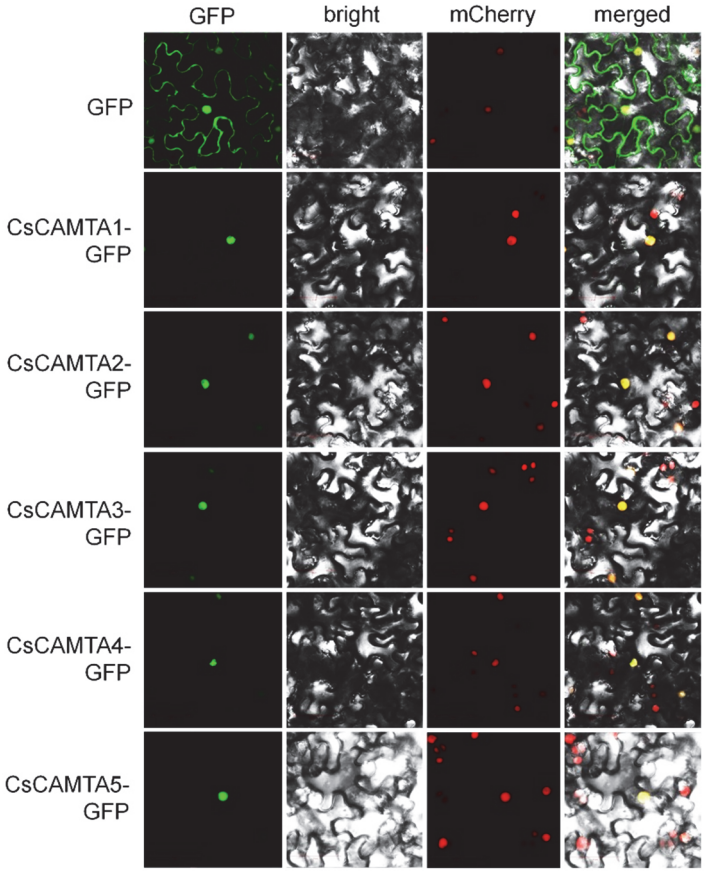

Fig. 6. Localization of calmodulin-binding transcription activator (CsCAMTA) proteins to the nucleus. *Nicotiana benthamiana* leaves were transiently transformed by agroinfiltration with constructs harboring pFGC-Egfp:CsCAMTAs or pFGC-Egfp. Fluorescence of target proteins was observed with confocal microscopy after 48 h.

The CAMTAs are likely found exclusively in multicellular organisms suggesting their involvement in cell-cell communication and developmental processes (Bouché *et al*. 2002). In mammals, CAMTAs are involved in controling growth and cell proliferation (Song *et al*. 2006). However, no direct evidence has been provided for the role of the plant CAMTA family in these processes. Yang *et al*. (2012) reported that *SlSR2* is scarcely expressed at two critical developmental stages of tomato fruit, whereas *SlSR3L* and *SlSR4* are expressed in fruit tissues exclusively. Moreover, *SlSR2L* expression is dramatically altered in a ripening mutant *rin* compared with a wild type fruit. In *Gossypium hirsutum*, *GhCAMTA2A.2* and *GhCAMTA7A* have high expressions in initiation and secondary cell wall synthesis stages, respectively. Additionally, several genes encoding, *e.g*., fatty acid desaturase, fatty acid desaturase linked oxidase, kinesin, protein kinase, auxin/indole-3-acetic acid (AUX/IAA) protein, ABC transporter-like, DNA-binding WRKY, MYB, homeodomain, zinc finger, leucine-rich repeat, and cellulose synthase protein families, which have imperative roles in cotton fiber development, are positively or negatively co-expressed with *GhCAMTA2A.2* and *GhCAMTA7A* (Pant *et al*. 2018). In the present study, the expressions of all the five *CsCAMTAs* were lower in mature fruit peel and pulp than in early fruit development

and ripening stages except the expression of *CsCAMTA1* (Fig. 2). Collectively, these data indicate that citrus *CAMTA* genes may be involved in regulating citrus fruit development and ripening processes.

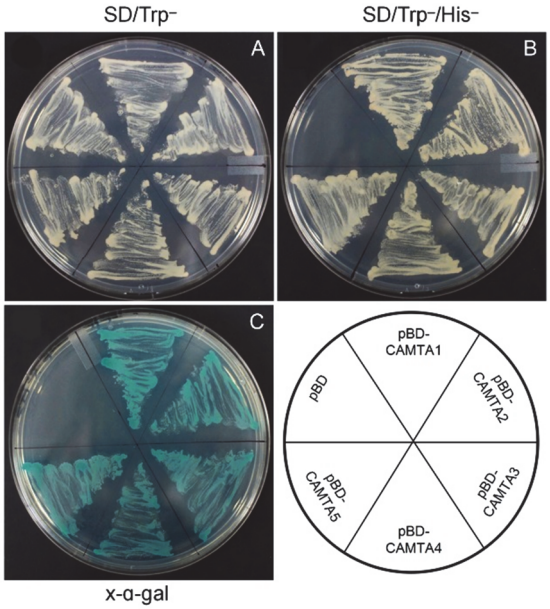

Fig. 7. The activity of calmodulin-binding transcription activator (CsCAMTA) proteins in yeast. The panels in *A* and *B* indicate the growth of yeast cells carrying pBD-CsCAMTAs and pBD (as a negative control), on SD/-Trp and SD/-Trp-His media, respectively, and the panel *C* shows results of β-alactosidase activity assay.

Drought, salt, low temperature, and heat stress can result in water deficit in plant tissues (Aroca *et al.* 2007, Wahid and Close 2007). The *CAMTA* gene expressions can respond to these abiotic stresses in many species (Pandey *et al*. 2013). In *Arabidopsis*, *CAMTA1* shows an increased sensitivity to drought with a reduced survivability (Pandey *et al*. 2013). Silencing *SlSR1L* in tomato leads to a decrease in drought stress tolerance, acceleration of leaf water loss, reduction of root biomass, and attenuation of expressions of drought stress responsive genes (Li *et al*. 2014). The *AtSR1/CAMTA3* demonstrates the role of *CAMTA* in salinity; a mutant shows more tolerance to salt stress because of the repressed expression of salt-responsive genes (Prasad *et al*. 2016). Many stress-responsive genes encode proteins to protect cells from a water stress and regulate gene expression for signal transduction in water stress response. The *AtCAMTA1* positively induces the expression of transcription factors (such as AP2, bZIP, MYB, WRKY, and GRAS) most likely by binding to CGCG and CGTG boxes in the promoter region of these target genes. The regulated expressions of these transcription factors restrict the expression of various genes and control regulatory proteins for maintaining the homeostasis of plants channelled through various adaptive and stress responsive genes (Pandey *et al*. 2013). The *AtCAMTA3* is a positive regulator of *C-repeat-binding factor 2* expression, and double *camta1*/*3* mutant plants are impaired in freeze tolerance, establishing a role for CAMTAs in the regulation of plant responses to cold (Doherty *et al*. 2009). The expressions of *CAMTAs* response to both environmental stresses (drought, salt, low temperature, and heat stresses) and ABA, by inducing a series of signalling cascades (Yue *et al*. 2015). The results presented in this study show that members of the *CsCAMTA* family were responsive to various environmental stresses (Fig. 3, 4, and 5). For example, the expression of *CsCAMTA5* was induced under all the tested stresses and the ABA treatment. However, the expressions of *CsCAMTA1* and *CsCAMTA3* were downregulated by the drought and the salt stresses but upregulated by ABA. The expression profiles of *CsCAMTA5* and *CsCAMTA3* were similar to the results observed in the *At/GmCAMTA* family and *ZmCAMTA4a*/*7b*, respectively (Yang and Poovaiah 2002, Wang *et al*. 2015, Yue *et al*. 2015). The expressions of *CAMTAs* in response to abiotic stresses and ABA indicate their important role in the signal transduction of plant water deficit by both ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki 2006). Furthermore, we observed the induction or the suppression of CsCAMTA family genes in response to tested stresses suggesting a crosstalk between different stress signalling pathways.

In addition, defence against different biotic stresses is specified by antagonism between the SA and JA/ET signalling pathways (Atkinson and Urwin 2012, Laluk *et al*. 2012). The ET and SA are often involved in CAMTA regulations of pathogenesis-related protein expression, leading to plant defence against pathogens (Dempsey *et al*. 2011). The *AtSR1*/*CAMTA3*, which are significantly up-regulated in response to MeJA, negatively regulates plant

defense against pathogens, insect attack, and wounding through JA- and SA-mediated pathways (Laluk *et al*. 2012, Qiu *et al*. 2012). By contrast, SA, MeJA, ET, and pathogen infection lead to up-regulation of *SlSR1* in tomato fruit, whereas *SlSR3L* is induced by SA, ET, and pathogen infection (Yang *et al*. 2012, 2013). Silencing *SlSR1* and *SlSR3L* in tomato confers an increased resistance to *B. cinerea* and *P. syringae* pv. *tomato* DC3000 through modulating the SA- and ET-signalling pathways (Li *et al*. 2014). In our study, SA and JA significantly induced the expressions of *CAMTA1*, *4*, and *5*. These findings are similar to those of previous studies in *Arabidopsis* and tomato (Yang and Poovaiah 2002, Yang *et al*. 2013) suggesting a potential role of *CAMTAs* in regulation of biotic stress.

Proteins targeted to the nucleus contain amino acid targeting sequences called nuclear localization signal NLS (Liu *et al*. 1999, Lange *et al*. 2007). The AtCAMTA1, 3, and 5 contain an NLS region, and they are located in the nucleus (Bouché *et al*. 2002, Yang and Poovaiah 2002, Mitsuda *et al*. 2003). Almost all the CAMTAs contain a NLS region in the CG-1 domain. We also found a NLS region in the CG-1 domain of CsCAMTAs (Fig. 1 Suppl.). In the present study, we examined the subcellular localization of CsCAMTAs, and the results indicate that the members of this family were nucleus-targeted proteins (Fig. 6), which is consistent with that of AtCAMTA1, 3, and 5. Additionally, transcription activation domains have been mapped in *Arabidopsis AtCAMTA1* and human *HsCAMTA2*, and both transcription activation domains map to a region between the CG-1 and TIG domains (Finkler *et al*. 2007). The *CAMTA* participates in transcriptional regulation of downstream genes by recognizing and binding to a specific *cis*-element (G/A/C)CGCG(C/G/T), and thereby leading to physiological responses (Yang and Poovaiah 2002, Galon *et al*. 2008, Leng *et al*. 2015, Shen *et al*. 2015). The *AtSR1/CAMTA3* bind to the core vCGCGb motif of several genes including *EDS1* (*enhanced disease susceptibility1*) and *NDR1* (*non-race-specific disease resistance1*) and the *EIN3* (*ethylene insensitive3*) promoter region *in vivo* (Doherty *et al*. 2009, Du *et al*. 2009, Nie *et al*. 2012). In our study, all the five *CAMTA* genes of citrus also acted as transcriptional activators (Fig. 7). This result correlates with a fact that tomato *SlSR1/3L*, *Arabidopsis AtCAMTA1/AtCAMTA5*, and human *HsCAMTA1/2* also function as activators (Bouché *et al*. 2002, Mitsuda *et al*. 2003, Li *et al*. 2014).

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