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ARTICLE

Functional analysis of tomato calmodulin gene family during fruit development and ripening

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Calmodulin is a ubiquitous calcium sensor to recognize the different developmental and/or stimulus-triggered calcium changes and regulate plant growth and development. However, the function of calmodulin remains elusive for fleshy fruit development. We performed expression studies of a family of six calmodulin genes (*SlCaMs*) in tomato fruit. All calmodulins showed a double peak expression pattern. The first flat peak appeared at 10–30 days after anthesis, but their expression rapidly declined at mature green and breaker. Then a sharp and even higher peak came at turning/pink stages. Among six calmodulins, *SlCaM1* had the highest expression during fruit enlargement, whereas *SlCaM2* was the major calmodulin during fruit ripening. However, *SlCaMs* showed different patterns in three ripening mutants *rin*, *Nor* and *Nr*. In particular, at the stages corresponding to mature green and breaker, the expression levels of *SlCaMs* in those mutants were significantly higher than wild-type. Furthermore, *SlCaMs*, especially *SlCaM2* were upregulated by ethylene. Transiently overexpressing *SlCaM2* in mature green fruit delayed ripening, while reducing *SlCaM2* expression accelerated ripening. Our results suggest that *SlCaMs* play double roles to regulate fruit ripening. Prior to the ethylene burst, the ethylene-independent repression of *SlCaMs* might be critical for fruit to initiate the ripening process. After the ethylene burst, *SlCaMs* could participate in the ethylene coordinated rapid ripening.

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INTRODUCTION

Fleshy fruits consist largely of soft succulent tissue, and contribute to a significant part of human diet for fibers, minerals and various nutraceuticals which are beneficial to human health. Right from the early stage of fruit set to ripening, fruit undergoes periods of cell division, cell expansion and finally ripening.[1,2] Postharvest perishability and quality depends on the control of fruit maturation/ripening process. Fruit ripening is a complex, yet genetically programmed process affected by external and internal cues.[3,4] Tomato (*Solanum lycopersicum*) is an excellent model to study climacteric fruit development and ripening due to its relatively short generation time, a long known history of physiological, biochemical and molecular investigations and advancement in the development of genetic and molecular tools for this species. The pleiotropic ripening mutants in tomato have added greatly to the understanding of ripening in fleshy fruit. These mutants include ripening-inhibitor (*rin*), non-ripening (*Nor*) and never-ripe (*Nr*). The *Rin* and *Nor* genes encode a MADS-box and a NAC-domain transcription factor, respectively, and it is suggested that they regulate both ethylene-independent and -dependent ripening processes.[5] *Nr* is directly involved in ethylene-dependent ripening because *Nr* encodes an ethylene receptor.[6] However, the details of a developmental regulatory cascade remain to be defined.[4,7]

It is established that the divalent ion calcium is important in controlling fleshy fruit ripening and shelf-life.[8–11] Calcium application can delay fruit ripening, and maintain fruit quality, in particular firmness, and thereby alleviate postharvest decay. It has been suggested that calcium acts through rigidifying the cell wall by cross-linking pectic acid residues[12] and stabilizing cell membranes.[13] Nevertheless, accumulating evidence indicate that calcium is a universal second messenger, and plays an important role in plant growth and development by mediating response to a variety of environmental and hormonal signals.[14–16] Signal triggered intracellular calcium changes are perceived by calcium sensors. Calmodulin is a ubiquitous calcium sensor, and present in all plant cell types examined thus far.[17–20] It can modulate the actions of diverse target proteins involved in almost all aspects of cell activity including cell division, cell elongation, ion transport, secondary metabolism, plant defense, *etc.*[17–20]

Based on genome sequence analysis, tomato has six calmodulin genes (*SlCaMs*), which are expressed in all organs, including fruits.[21] *SlCaMs* in vegetative tissues were highly responsive to a variety of biotic and abiotic stimuli such as ethylene, abscisic acid, benzothiadiazole and jasmonic acid. Silencing of *SlCaM2* and *SlCaM6* altered expression of defense-related genes and reduced resistance to pathogens. Accumulation of calmodulin mRNA and protein were also observed in tomato leaves after wounding or systemin treatment.[22] In addition, several calmodulin-target proteins have been reported in tomato fruits. For example, calmodulin can increase the activity of glutamate decarboxylase. Glutamate decarboxylase is a key enzyme for the biosynthesis of gamma-aminobutyric acid,[23,24] a beneficial compound to human health.[25] SUN belongs to the IQD family of calmodulin-binding proteins[26,27] and controls fruit elongation.[28] Recently, we characterized a family of calmodulin-regulated transcription factor family SlSRs during tomato fruit development.[29,30] All *SlSRs* were highly yet differentially expressed during fruit development and ripening. Most notably, the expression of *SlSR2* was scarcely detected at the mature green and breaker stages, two critical stages during the transition from fruit size enlargement to fruit maturation. The expression profiles of all seven

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*SISR*s were altered in ripening mutants compared with wild-type fruit. However, so far, it is still not clear how and whether calcium/calmodulin signaling is involved in fruit ripening. Here we report an expression analysis of the calmodulin gene family in wild-type tomato and ripening mutants, as well as the functional significance of a specific calmodulin gene to regulate fruit-ripening process.

MATERIALS AND METHODS

Plant materials

Tomato plants (*S. lycopersicum* cv 'Rutgers' and 'Moneymaker') were grown in greenhouse at 28 ℃ and 16 h/8 h (light/dark). 'Rutgers' was used for gene expression studies, whereas 'Moneymaker' was mainly used for functional studies by transient transformation. The ripening mutants *rin*, *Nor* and *Nr* were all in 'Rutgers' background. Tomato fruit development stages were divided into fruit enlargement stages counted by days post anthesis (DPA) and the visual color changes during development and ripening set by USDA (http://www.ba.ars.usda.gov/hb66/tomato.pdf). Mature green fruit was the first stage after the maturation of the seeds. At this stage, the fruit was fully expanded with a green surface. Breaker stage fruit was defined when first visible sign of carotenoid accumulation was evident. This stage was followed by, turning, orange, red ripe and over-ripe stages. The equivalent stages to mature green, breaker and red stages for three ripening mutants are 45, 50 and 65 DPA, respectively. The pericarp color of those mutants keeps green before 50 DAP. At 65 DPA, their fruit surfaces show the different degrees of yellowness. Leaves and roots were collected from the third fully opened leaves from top and 20 mm long roots counting from root-tip of 3-month old plants, respectively. Flowers were the whole immature buds (~12 mm).

Ethylene treatment

The greenhouse-grown fruits at the mature green stage were held under ambient conditions overnight to reduce harvest shock prior to treatment. Thereafter the fruit were sealed in a jar with 100 ppm ethylene for different time periods. Pericarp tissue excised from the fruit was frozen in liquid nitrogen and stored in −80 ℃.

RNA extraction and RT-qPCR

Total RNA was isolated from frozen tissue using RNeasy Plant Mini Kit following the manufacturer's instruction (Qiagen, Valencia, CA, USA). Reverse transcription and qPCR were performed as described [29]. Briefly, one μg of total RNA was used to synthesize cDNA with iScript™ kit (Bio-Rad, Hercules, CA, USA), RT-qPCR analysis of cDNA was performed on a CFX96 Real-time System (Bio-Rad). Gene specific primers listed in Table 1 were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3/). Relative fold difference (*N*) was the number of the treated target gene copies relative to the untreated control gene copies and is calculated as follows: $N=2^{\Delta Cq}$. ΔCq was the difference in threshold cycles for *SlCaMs* targets and the *actin* internal reference. Relative gene expression (fold changes) was calculated based on *N* with the lowest value as 1. Student's *t*-test ($p<0.05$) was used to determine the significant difference of relative expression of individual genes among different treatments and controls (Microsoft Excel 2007). The results are based on at least three repeats in three independent experiments.

Construction of Ti plasmids carrying sense- and antisense- *SlCaM2*

Full length *SlCaM2* were amplified from a mixture of fruit tissues by Pfx DNA polymerase, and subcloned to TA cloning Kit (Life Technology, Grand Island, NY, USA) using gene-specific primers (Table 1). The nucleotide sequences of the positive clones were confirmed by sequencing. The full length of *SlCaM2* were subcloned into pDL198,[31] a derivative of pCambia1300 in either sense- and antisense- orientations in the sites of *Kpn* I and *Bam*H I downstream of 35S promoter, and introduced into *Agrobacterium tumefaciens* strain GV3101. The positive clones were verified by PCR using gene-specific primers.

Fruit agroinfiltration

Agroinjection of tomato fruit was carried as described by Orzaez *et al*.[32] Briefly, *Agrobacterium* cultures were grown overnight from individual colonies at 28 ℃ in YEB medium plus selective antibiotics, transferred to induction medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mm $MgSO_4$, 20 μm acetosyringone, 10 mm MES, pH 5.6) plus antibiotics, and grown again overnight. The next day, cultures were resuspended with infiltration medium (10 mm $MgCl_2$, 10 mm MES, 200 μm acetosyringone, pH 5.6 with OD_{600} of 1.0), and incubated at room temperature with gentle agitation (20 r.p.m.) for a minimum of 2 h. Cultures were injected into the early mature green fruits which were still attached to plants using a syringe with a 0.5-mm×16-mm needle. The needle was inserted into the fruit tissue through the blossom end. Because of differences in fruit size, the injection was terminated when the solution started running out the injection site. The agroinfiltrated fruits continued the developmental process *in planta*.

Table 1 Primers used for qRT-PCR and cloning

Primer name	Oligonucleotides	Gene ID
SlCaM1-a	CCA GAG TTC CTT AAC CTG ATG G	Solyc01g008950
SlCaM1-b	CTT TTC GCC TAG GTT TGT CAT C	
SlCaM2-a	TCT GAG GAG GAG TTG AAA GAG G	Solyc10g081170
SlCaM2-b	TCA ACA TCA GCT TCC CTA ATC A	
SlCaM3-a	GAT GGT AAT GGA ACC ATC GAC T	Solyc10g077010
SlCaM3-b	CAT CAG TGA GCT TCT CAC CAA G	
SlCaM4-a	TCA GAT CTC GGA GTT CAA AGA AG	Solyc11g072240
SlCaM4-b	CAG GTT AAG GAA CTC AGG GAA GT	
SlCaM5-a	TTA ACT TGA TGG CTC GGA AGA T	Solyc12g099990
SlCaM5-b	ACG AAT CAT CTC GTC AAC CTC T	
SlCaM6-a	ATC ACT TGG TCA GAA TCC CAC T	Solyc03g098050
SlCaM6-b	AGC TGC AGA AAT AAA GCC ATT C	
SlE4-a	ACC AGC AAT ATC TAG AGA AGG GTG	Solyc03g111720
SlE4-b	ATC ATT GTC ATG TTT ATT CAA ATT TAA AG	
SlActin-a	GAA ATA GCA TAA GAT GGC AGA CG	X55749
SlActin-b	ATA CCC ACC ATC ACA CCA GTA T	
SlCaM2-S1*	ggt ggt accATG GCG GAT CAG CTG ACG G	
SlCaM2-S2*	gga gga tcc CTT GGC CAT CAT GAC CTT AAC	
SlCaM2-A1*	ggt ggt accCTT GGC CAT CAT GAC CTT AAC	
SlCaM2-A2*	gga gga tccATG GCG GAT CAG CTG ACG G	

* Primers used for cloning *SlCaM2* into the plant transformation vectors. The underlined are the restriction sites.

Confocal laser scanning microscopy

Thin slices of tomato fruits were placed in cover glass bottom petri dishes (MatTeck Corp., Ashland, MA, USA) for observation. A Zeiss™ LSM710 confocal laser scanning microscopy (Carl Zeiss Microscopy, Thornwood, NY, USA) system was utilized. The images were observed using a Zeiss Axio Observer™ inverted microscope with a 10×0.45 NA Plan Apochromatic objective. Both differential interference contrast and fluorescence images were acquired simultaneously. A photomultiplier tube captured the light emitted from a 488 nm argon laser with a pin hole of 3.7 μm passing through a MBS 488 filter with limits set between 492 and 543 nm. Zeiss Zen™ 2012 software was used to obtain 15–20 z-stack images to produce the 3D renderings, which were used to develop the 2D maximum intensity projections for publication.

Colorimetric analysis

A Minolta Chroma Meter CR-300 (Minolta Corp., Osaka, Japan) was used to assess the color of fruit surface. Three measurements per fruit and six fruits per treatment were taken. The means of value a^* and b^* were used to calculate the hue angle ($Hue=\arctan(b^*/a^*)$) and metric chrome value ($Chroma=(a^{*2}+b^{*2})^{1/2}$). Student's *t*-test ($p<0.01$) was used to determine the significant difference of different colorimetric parameters. The Chroma meter was calibrated with a standard white plate ($Y=94.00$, $x=0.3158$, $y=0.3322$).

RESULTS

Expression pattern during fruit development and ripening

During tomato fruit development, all six calmodulin genes in the pericarp showed a double peak expression pattern (Figure 1). The first peak was flat and appeared between 10 and 30 DPA when the fruit size was expanding. Then the expression levels for calmodulins dropped to the lowest at mature green and/or breaker. After that, a sharp peak was observed at turning stage and started to decline after orange stage. As the fruit became red, their expression levels

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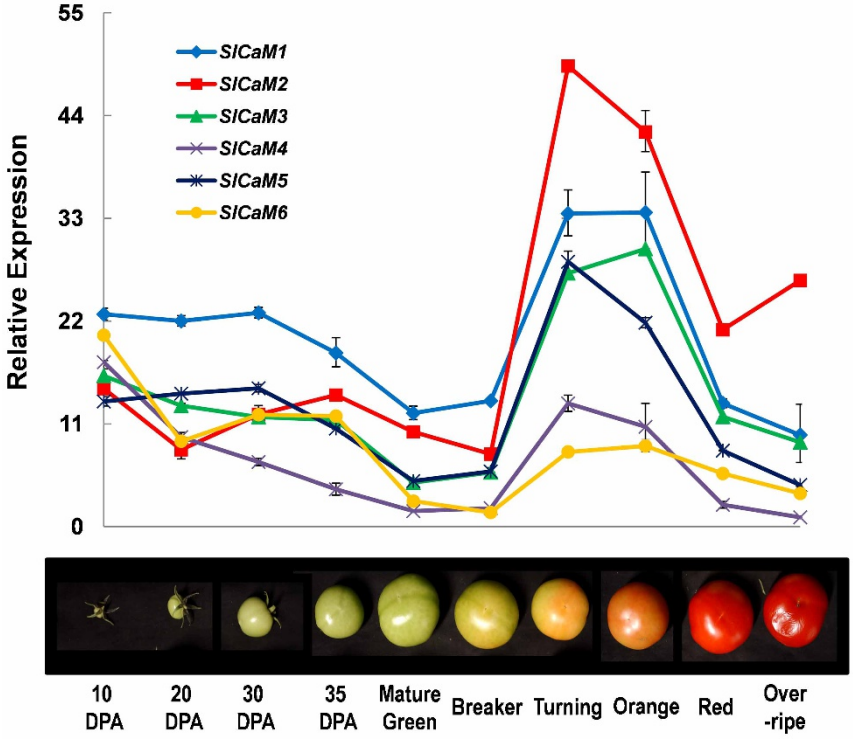

Figure 1. Expression patterns of *SlCaM* genes during fruit development. Relative gene expression levels are shown following normalization with actin transcript values. Error bars represent standard error of the mean. For each gene, different letters indicate significant differences among mean values ($p<0.05$). The bottom shows the fruit images at 10, 20, 30, and 35 days post anthesis (DPA), mature green, breaker, turning, pink, red and overripe stages. The results are based on at least three repeats in three independent experiments.

declined rapidly. At over-ripe stage, the expression levels of all *SlCaMs* except *SlCaM2* dropped to the bottom basal level. The most abundant calmodulin in unripe fruit (from 10 DPA to breaker stage) was *SlCaM1* which had over 22-fold more than the lowest expression by *SlCaM6* at breaker stage. However, after breaker stage, the expression of *SlCaM2* was significantly enhanced, and reached as high as ~46-fold at turning stage. Thus *SlCaM2* became the most abundant calmodulin during fruit ripening, followed by *SlCaM1*, *SlCaM3* and *SlCaM5*. *SlCaM4* and *SlCaM6* had the lowest expressions at most stages. Their second peak at turning stage was even lower than their first peak. However, the other four calmodulins had the highest expression at turning stages when their expression levels were doubled or tripled as compared to their first peak occurring during fruit enlargement. These results suggest that higher expression of *SlCaMs* is required for both fruit enlargement and ripening. However, during the transition period, i.e., mature green and breaker stages, the suppression of *SlCaMs* was needed.

Expression in non-fruit tissues

To investigate whether any *SlCaMs* show the specific expression in fruit, we examined the expression level of *SlCaMs* in non-fruit tissues, including leaves, roots and flowers. Since the highest expression level of most *SlCaMs* in fruit occurred at turning stage, this stage fruit was added for comparative studies. As shown in Figure 2, *SlCaMs* were detected in all non-fruit tissues. As compared to the turning stage fruits, all *SlCaMs* had the comparable expression in roots, but much lower expression in flowers and leaves. In particular, leaves had the lowest expression for all *SlCaMs*, which were usually less than 10% of the corresponding mRNA levels in either roots or fruits. Relatively, *SlCaM1* was the most abundant in roots where *SlCaM2* had the lowest expression. These results suggest that *SlCaM2* could be a fruit preference calmodulin gene.

Expression of *SlCaM*s are changed in tomato ripening mutants

To determine whether the reduced expression of *SlCaMs* at mature green and breaker stages has any relationship to fruit ripening, we compared the expression levels of *SlCaM*s in the fruits from wild-type and three ripening-mutants, *rin*, *Nor* and *Nr* (Figure 3). First, we

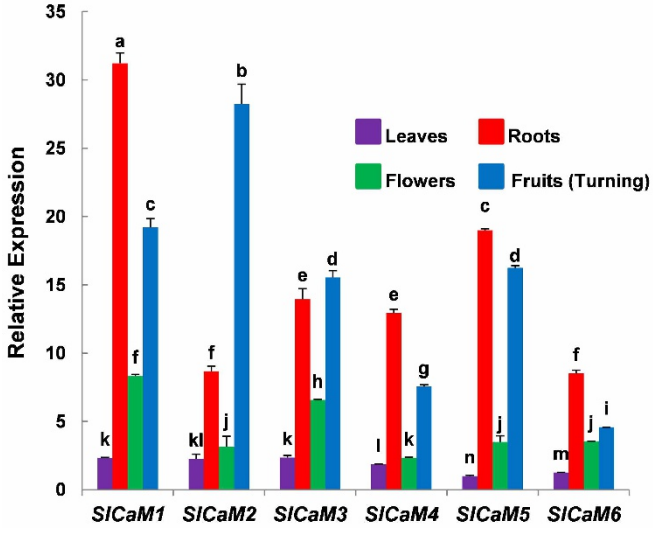

Figure 2. Expression patterns of *SlCaM* genes in non-fruit tissues. Relative gene expression levels are shown following normalization with actin transcript values. Error bars represent standard error of the mean. For each gene, different letters indicate significant differences among mean values ($p<0.05$). The results are based on at least three repeats in three independent experiments.

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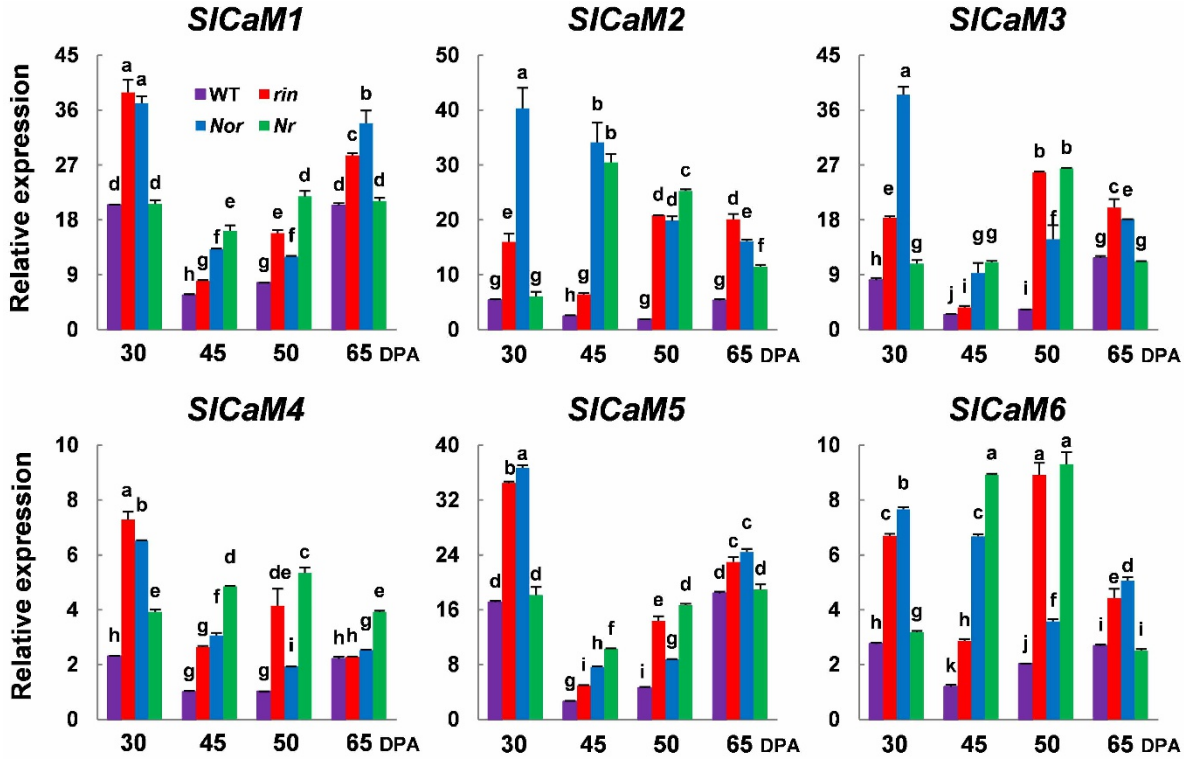

Figure 3. Expression patterns of *SlCaMs* in tomato ripening mutant fruits. Relative gene expression levels are shown following normalization with actin transcript values. Error bars represent standard error of the mean. For each gene, different letters indicate significant differences among mean values ($p<0.05$). The results are based on at least three repeats in three independent experiments.

analyzed the expression of *SlCaMs* at 45 and 50 DPA in these mutants which corresponded to the mature green and breaker stages in wild-type fruit. Interestingly, all three mutants, particularly *Nor* and *Nr* showed significantly higher expression for all *SlCaMs* at both stages. At 45 DPA, the most abundant calmodulin gene in *Nor* and *Nr* was *SlCaM2*. Its expression level in *Nor* and *Nr* was about 35- and 30-fold higher than wild-type, respectively. At 50 DPA, the expression levels of *SlCaM2*, *SlCaM3* and *SlCaM1* were the top three. We further examined the expressions in those mutant fruits at earlier stage (30 DPA) and later stage (65 DPA, equivalent to red stage in wild-type). In most cases, *SlCaMs* in three mutants had higher expression than those in wild-type. Occasionally, some genes, e.g., *SlCaM1*, *SlCaM3*, *SlCaM5* and *SlCaM6*, in some mutants, e.g., *Nr*, showed the comparable expression level to wild-type. Thus generally speaking, the expression level for *SlCaMs* were high in all ripening mutants at all stages. However, the expression patters for most *SlCaMs* in mutants were low at mature green and/or breaker stages; a double peak phenomenon was observed in wild-type. Nevertheless, the expression of *SlCaM2*, *SlCaM4* and *SlCaM6* was exceptional in *Nor* and *Nr*. For instance, *SlCaM2* displayed a gradual decrease from 30 DPA to 65 DPA in *Nor*. *SlCaM4* had no much change for all the four stages in *Nr*, whereas *SlCaM6* displayed higher expression in *Nr* at 45 and 50 DPA than at 30 and 65 DPA.

*SlCaM*s are ethylene responsive genes

Tomato fruit ripening is correlated to ethylene level. Nearly no endogenous ethylene was produced at mature green stage. Ethylene increased at breaker stage, and reached maximum at orange stages.[7] Thus, we selected mature green stage fruits for ethylene treatment in order to define whether all *SlCaM* genes in fruits are responsive to ethylene. All *SlCaM*s showed positive responses to ethylene treatment as early as 1 h after treatment, and peaked at 4 h after treatment (Figure 4). In particular, *SlCaM2* exhibited the highest response to ethylene. Ethylene triggered *SlCaM2* over ~15-fold and ~45-fold at 1 and 4 h after treatment. In comparison, the expression levels of other *SlCaMs* were boosted three- to fivefold by ethylene at four hours after treatment. *E4* was a defined ethylene responsive gene,[33] and showed dramatic response to ethylene treatment, which indicated that ethylene treatment was successful. These results demonstrate that all *SlCaM*s, in particular *SlCaM2*, are ethylene responsive genes.

Alteration of *SlCaM2* level affects fruit ripening

Based upon the expression patterns of *SlCaMs* during fruit development in wild-type and ripe mutants and their response to ethylene treatment, it seemed that *SlCaM2* could be a stronger candidate to function during fruit ripening. Thus, we selected it for further functional analysis *in planta*. To quickly assess the transgene expression in fruit, the agroinjection method was utilized to transiently express the candidate genes in the early mature-green fruit. We first injected *Agrobacterium* cultures carrying a control vector (pDL198) containing *35S: GFP* into fruit. Green fluorescence was detected after 36 h, and reached a maximum at 48 h (Figure 5a). After that, the green fluorescence gradually faded, and completely disappeared at day 6. However, no green fluorescence was detected in the fruit injected with *Agrobacterium* only. These results indicate that the transgene can be temporally highly expressed at the late mature green stage when agroinfiltration is performed in early mature green fruit.

Similarly, *SlCaM2*- sense and antisense constructs were agroinjected into early mature green fruits. Ninety-six hours after injection (day 4), the expression level of *SlCaM2* in control (pDL198 vector alone) was about twofold higher than non-agroinfiltrated fruit (wild-type) (Figure 5b), suggesting that *Agrobacterium* itself slightly stimulated the endogenous *SlCaM2* expression. However,

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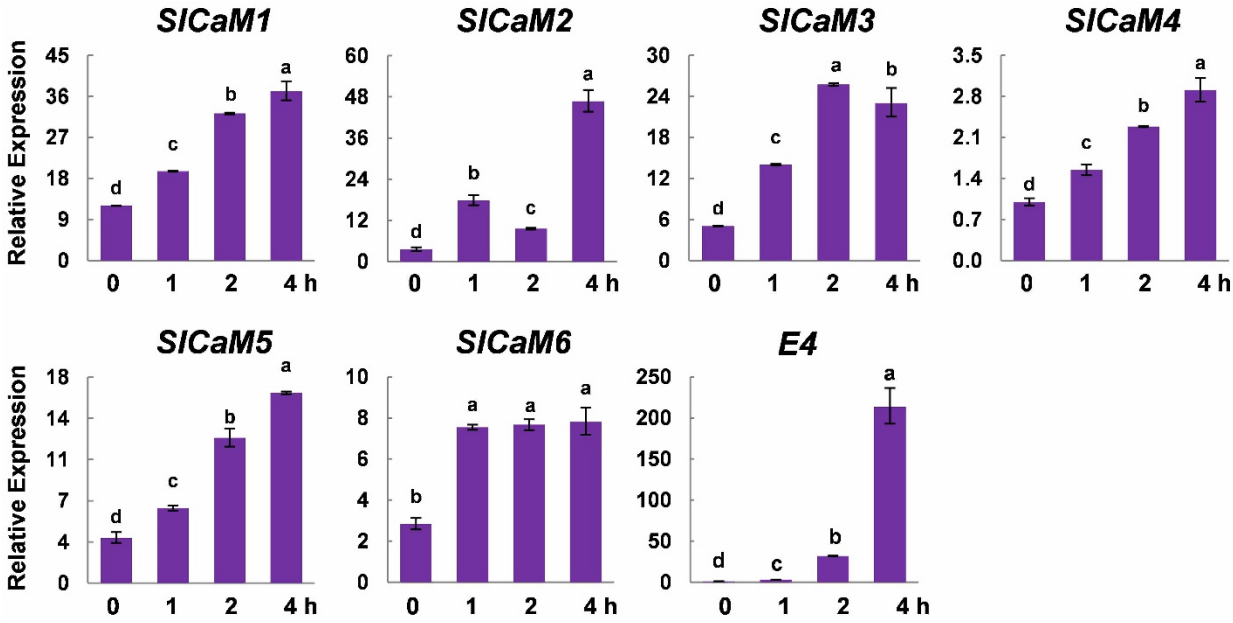

Figure 4. *SlCaMs* expression levels are stimulated by ethylene treatment. Tomato fruits (cv. Rutgers) at mature green stage were treated with 100 ppm ethylene for different time periods as indicated. Relative gene expression levels are shown following normalization with actin transcript values. Error bars represent standard error of the mean. For each gene, different letters indicate significant differences among mean values ($p<0.05$). The results are based on at least three repeats in three independent experiments.

as compared to the vector alone control, *SlCaM2* in the sense fruit was increased by over fivefold at day 4. In contrast, the expression of *SlCaM2* in the antisense fruit was reduced by 4–5 times. In addition, no significant change in the level of other calmodulins was detected in the sense and control fruit. Instead, the expression levels of other calmodulins in the antisense fruit were also reduced by 1.6- to 3.6-fold, suggesting that the antisense *SlCaM2* can reduce total level of calmodulins, too (data not shown), due to the high homology between all calmodulins. On day 6, the antisense fruits began the color change from green to white, while no obvious color change was observed in control and sense fruits until days 8 and 9. On day 11, the antisense fruit turned to orange/red (Figure 5c, when wild-type fruit (without agroinfiltration) reached turning/orange stage (data not shown). In comparison, the vector alone control fruit reached breaker/turning, and sense fruit was at late breaker (Figure 5c). The difference of fruit surface color on day 11 was further analyzed by a colorimeter (Table 2). There were no much differences in b^* value in all types of fruits. The antisense fruits had a slightly lower L^* and higher Chroma value than other fruits. However, the significant difference ($p<0.01$) in a^* value was detected between the antisense fruits and sense fruits. The antisense fruits had a^* value of 20.6, whereas a^* value for the sense fruits was −5.3. Accordingly, the Hue value for the antisense fruit was 55.9, much lower than the sense fruits (99.3). As for the control (*35S: GFP*) fruits and wild-type fruits (no agroinfiltration), their a^* value and hue were 1 and 87.9, 5.5 and 79, respectively. Thus the fruit surface color of the antisense fruits was much redder than others, while the sense fruits were more greenish than all other fruits including the control fruits.

Further gene expression examination at day 11 showed that the expression levels of *SlCaM2* in all fruits were increased as compared to those at day 4. However, *SlCaM2* in the antisense fruit was increased by 40- to 50-fold, whereas its levels in control and sense fruit were increased by ~21- and ~9-fold, respectively (Figure 5b). The expression of ethylene-responsive gene *E4* in the antisense fruit showed 38 and 105 times higher than the control fruit and sense fruit at day 11, respectively (data not shown), suggesting that there was more endogenous ethylene produced in the antisense fruit. These results indicate that temporally reducing *SlCaM2* (maybe total calmodulins) at mature green can facilitate fruit ripening.

DISCUSSION

Tomato fruit development includes two periods, fruit enlargement and fruit ripening.[1,3] Fruit enlargement results from both cell division and cell elongation. After fruit reaches the maximum size at mature green, the ripening process starts at breaker as shown by the degradation of chlorophyll and formation of carotenoids. In this study, we analyzed the gene expression profiles of the calmodulin gene family during fruit development, and found that the expression of all the calmodulins exhibited a double peak pattern, one peak at fruit enlargement stages, and another during fruit color changes from mature green to red (Figure 1). It is well documented that calcium/calmodulin is critical for cell division because the distribution of calmodulins is highly located in proliferating cells such as root tip, young shoot and developing embryos/seed, but low in expanding leaves and the base of stems.[34–37] Calmodulin also appears high in cells with specialized events and signaling, such as pollen tube growth, cotton fiber elongation and coleoptiles elongation.[38–40] In tomato, SUN, a calmodulin-binding IQD family protein is shown to control fruit elongation.[28] Hence high calmodulin expression during fruit enlargement might contribute to promote cell division and cell elongation. Once the fruit reached the maximal size at mature green, the level of calmodulin rapidly decreased (Figure 1). All these results suggest that calmodulins play a role for fruit size increase.

It was expected that the calmodulin expression is kept at a low level at breaker when fruit changes color due to the chlorophyll breakdown. Calcium treatment has been shown to delay the leaf senescence.[41,42] Knockouts of calcium/calmodulin-binding proteins such as SR1/CaMTA3, CNGC and MLO showed early leaf senescence and chlorosis.[43,44] Thus the low expression of calmodulin in breaker fruit could result to the chlorophyll degradation. However, after breaker, expression of calmodulins was

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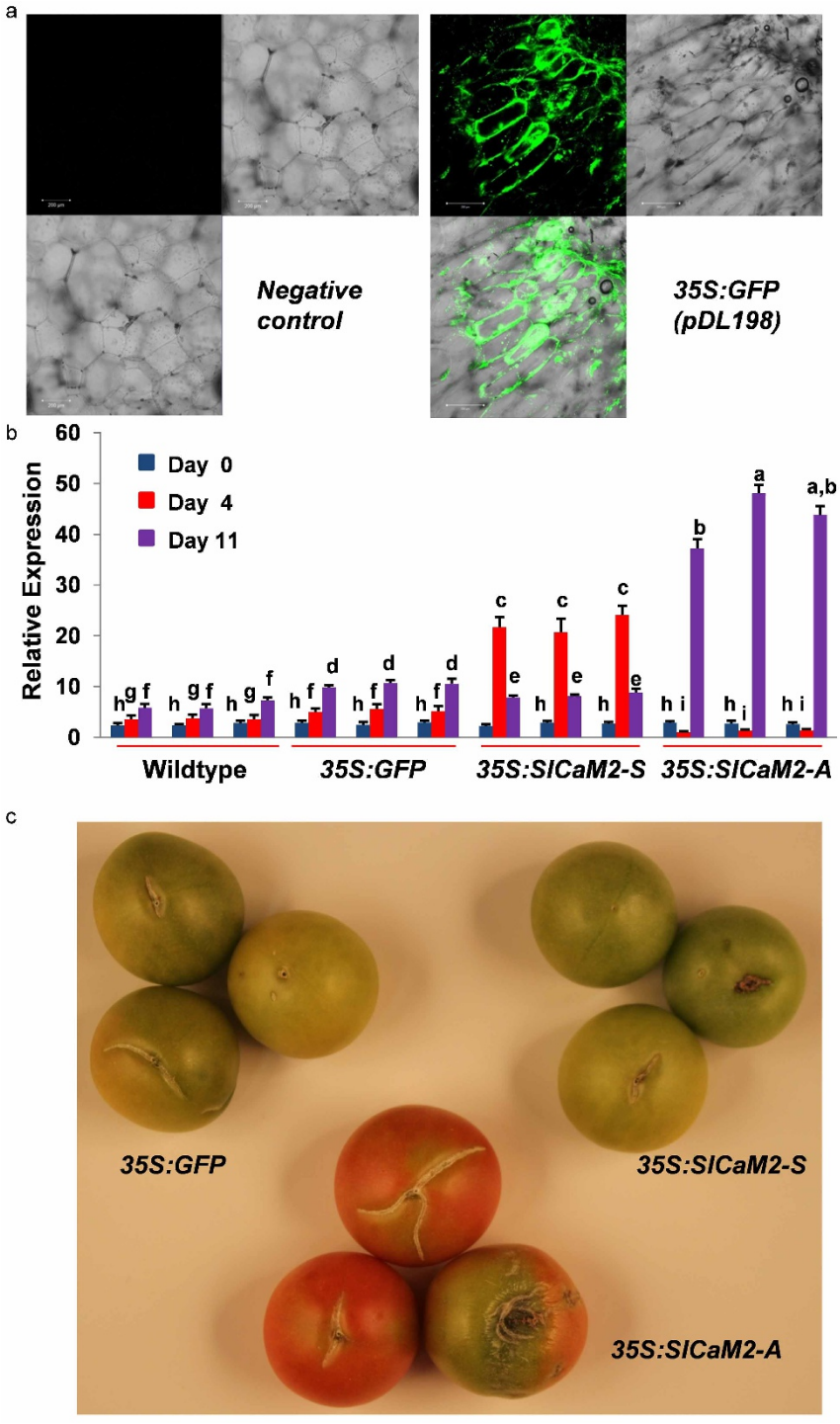

Figure 5. Expression level of *SlCaM2* in fruit affects fruit ripening. The early mature green fruit were agroinfiltrated with *Agrobacterium* carrying different constructs. *35S: GFP*, pDL198 empty vector (control); *35S: SlCaM2-Sense*, pDL198 carrying *SlCaM2* in the sense orientation downstream of 35S promoter; *35S: SlCaM2-Antisense*, pDL198 carrying *SlCaM2* in the antisense orientation downstream of 35S promoter. (**a**) Confocal image of green fluorescence protein in agroinjected fruit tissue. The images were taken 48 h after agroinjection (day 4). Bar=200 μm. Negative control, agroinfiltrated with *Agrobacterium* only. (**b**) Examination of expression levels of *SlCaM2* in different agroinfiltrated fruits. Days 0, 4 and 11 after agroinfiltration, a piece of pericarp tissue from each fruit was used for RT-qPCR analysis. Relative gene expression levels are shown following normalization with actin transcript values. Different letters indicate significant differences among mean values ($p<0.05$). (**c**) Fruits at Day 11 after agroinfiltration show the difference in fruit color and maturity after agroinfiltrated with different constructs.

dramatically stimulated and reached the second peak at pink stage when they were even higher than the first peak during fruit enlargement (Figure 1). This could be mainly stimulated by ethylene burst occurring after late breaker[7,45] since all calmodulins were ethylene-responsive (Figure 4). These results suggest that calmodulins acts in the ethylene-dependent ripening process.

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Table 2 Color parameters and ripe indexes for agroinfiltrated fruits (Day 11)

Fruits[1]	*L**	*a**	*b**	Hue	Chroma
Wild-type	52.4±0.6[a]	5.5±0.8[c]	28.5±1.2[a]	79.0±1.8[c]	29.0±1.1[a]
35S:GFP	53.1±0.4[a]	1.0±0.3[b]	29.3±0.6[a]	87.9±0.7[b]	29.3±0.6[a]
35S:SlCaM2-S	54.6±1.0[a]	−4.6±0.7[a]	28.4±1.2[a]	99.3±1.2[a]	28.8±1.1[a]
35S-SlCaM2-A	47.9±1.1[b]	20.5±1.2[d]	30.4±0.8[a]	55.9±1.0[d]	36.7±1.3[b]

[1] Wild-type: no agroinfiltration; *35S:GFP:* pDL198 empty vector; *35S:SlCaM2-S*: pDL198 carrying *SlCaM2* in the sense orientation downstream of 35S promoter; *35S:SlCaM2-A*, pDL198 carrying *SlCaM2* in the antisense orientation downstream of 35S promoter.

Nevertheless, our data indicate that calmodulins may play an important role to regulate fruit ripening upstream of ethylene signaling, too. First, calmodulin levels in three ripening mutants were significantly higher than wild-type in all the examined stages. In particular, *SlCaMs* in those mutants did not show the obvious double peak pattern (Figure 3). Instead, all the three ripening mutants had the significantly high expression of *SlCaMs* at the stages corresponding to mature green and breaker in wild-type fruit. Second, temporally overexpressing *SlCaM2* in the mature green fruit delayed the maturation process, while repressing *SlCaM2* led to the accelerated ripening (Figure 5). Hence, the reduced expression of calmodulins at mature green and breaker could be a prerequisite for fruit to enter the ripening process. If the calmodulin level cannot be reduced at those stages, fruit cannot initiate the ripening process as shown in ripening mutants. Notably the similar expression pattern was observed in a calmodulin-regulated transcription factor *SlSR2*,[30] and β-galactosidase gene *TBG5* at the same stages.[46] β-galactosidase is important for hemicellulosic modifications that occur during cell division, cell growth and fruit ripening. Further studies are necessary to define whether they are the direct and/or indirect downstream targets of *SlCaM2*.

Calmodulin belongs to a small gene family in plants. Multiple calmodulin genes often show differential expression in tissues, developmental stages, and in response to numerous external and internal stimuli such as temperature changes, light and pathogen attacks, and to hormones such as abscisic acid and ethylene.[16–19] It is believed that individual calmodulin genes could have unique functional application and significance.[17,47] For instance, *Arabidopsis* contains seven calmodulin genes encode four highly conserved isoforms. A loss-of-function mutant in *AtCaM2* affects pollen germination.[48] However, the *atcam3* knockout mutant exhibits reduced thermotolerance after heat treatment, whereas overexpressing *AtCaM3* significantly increases thermotolerance.[49] Tomato has six calmodulins encoding four isoforms.[21] In our study, *SlCaM2* showed the most dramatic changes during the transition period from mature green to ripe (Figure 1) and the most responsive to ethylene treatment (Figure 4). The manipulation of its expression level during this period clearly affected the fruit maturation process (Figure 5). Thereby, among all the calmodulins, *SlCaM2* could be a major player to regulate fruit ripening, especially the ethylene-coordinated fruit ripening, although we cannot exclude the possible role of other calmodulins.

In conclusion, we have investigated six calmodulin expression profiles in tomato wild-type and ripening mutants during fruit development, and carried out the functional studies of *SlCaM2* during the transition period from mature green to ripe. Based upon our data, we propose a model to describe *SlCaMs*' role during fruit development and ripening (Figure 6). The expression pattern of *SlCaMs* is regulated by development signals. During fruit enlargement, highly expressed *SlCaMs* could contribute to cell division and elongation. After mature green, *SlCaMs* could play double roles to regulate fruit ripening. Prior to the ethylene burst, the downregulation of *SlCaMs* is required for fruit to initiate the ripening process. This process is ethylene-independent and may be present in both climacteric and non-climacteric fruits. The details of the regulatory cascade in this process still like a ''black box''. After the ethylene burst, *SlCaMs*

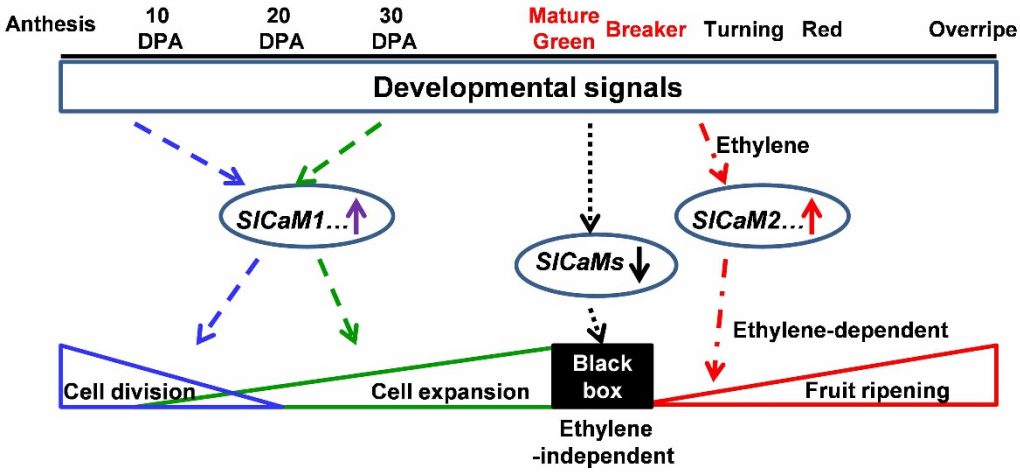

Figure 6. Schematic illustration of the proposed model for calmodulins' function during tomato fruit development and ripening. High expression of calmodulin during fruit enlargement could contribute to cell division and expansion. After ethylene burst, high calmodulin expression could be involved in ethylene-dependent ripening. Dowregulation of calmodulin during the transition period is critical for fruit to initiate the ripening (an ethylene independent event) although the mechanics is still not clear (like a 'black box'). The time line and stages may vary among different cultivars and different conditions.

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are rapidly stimulated by ethylene to participate in the ethylene coordinated rapid ripening. Further characterization of the *SlCaMs*-regulated signal transduction pathway in relation to ethylene-dependent and -independent ripenings and the ripening genes using the omics approaches[50] will shed light on the understanding of fruit ripening mechanics. Furthermore, it will be a great interest to determine whether the similar ethylene-independent regulation by calmodulin exists in other species, especially non-climacteric fruits.

COMPETING INTERESTS

The authors have no conflict of interest.

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