The calcium sensor GhCaM7 promotes cotton fiber elongation by modulating reactive oxygen species (ROS) production

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Key words: calcium signaling, calmodulin, feedback regulation, fiber elongation, Gossypium hirsutum (cotton), reactive oxygen species (ROS).

Introduction

Ionic calcium (Ca\(^{2+}\)) has been firmly established as a ubiquitous intracellular second messenger in plants (Sze et al., 2000; Hetherington & Brownlee, 2004; Hepler, 2005). Ca\(^{2+}\) signaling plays crucial roles in a wide array of growth and developmental processes (Jones & Lunt, 1967) and is especially important in tip growth (Fan et al., 2004; Yoon et al., 2006; Kudla et al., 2010). The tip-high Ca\(^{2+}\) gradient is coupled to an influx of Ca\(^{2+}\) from the extracellular environment that occurs predominantly in the apex (Hepler et al., 2001; Holdaway-Clarke & Hepler, 2003), and maintains the rate of cell elongation.

Calmodulin (CaM) is a highly conserved regulatory Ca\(^{2+}\) sensor that is ubiquitous in eukaryotic cells (Snedden & Fromm, 2001) and has a large number of physiological functions in animals and plants, although CaM has no catalytic activity of its own (Yang & Poovaiah, 2003). Its regulatory activities are manifested by its ability to modulate target enzymes. Ca\(^{2+}\)/CaM signaling is reported to be involved in tip growth of the pollen tube (Ma et al., 1999; Rato et al., 2004). Pollen tube elongation can be inhibited by extracellular application of CaM antagonists such as trifluoperazine (TFP), calmidazolium, W-7, and W-12 (Picton & Steer, 1985; Rato et al., 2004; Chen et al., 2009).

Fiber elongation is the key determinant of fiber quality and output in cotton (Gossypium hirsutum). Although expression profiling and functional genomics provide some data, the mechanism of fiber development is still not well understood.

Here, a gene encoding a calcium sensor, GhCaM7, was isolated based on its high expression level relative to other GhCaMs in fiber cells at the fast elongation stage. The level of expression of GhCaM7 in the wild-type and the fuzzless/lintless mutant correspond to the presence and absence, respectively, of fiber initials.

Overexpressing GhCaM7 promotes early fiber elongation, whereas GhCaM7 suppression by RNAi delays fiber initiation and inhibits fiber elongation. Reactive oxygen species (ROS) play important roles in early fiber development. ROS induced by exogenous hydrogen peroxide (H\(_2\)O\(_2\)) and Ca\(^{2+}\) starvation promotes early fiber elongation. GhCaM7 overexpression shows increased ROS concentrations compared with the wild-type, while GhCaM7 RNAi fiber cells have reduced concentrations. Furthermore, we show that H\(_2\)O\(_2\) enhances Ca\(^{2+}\) influx into the fiber and feedback-regulates the expression of GhCaM7.

We conclude that GhCaM7, Ca\(^{2+}\) and ROS are three important regulators involved in early fiber elongation. GhCaM7 might modulate ROS production and act as a molecular link between Ca\(^{2+}\) and ROS signal pathways in early fiber development.

Summary

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The inhibition of Ca\(^{2+}\)/CaM signaling results in ultrastructural abnormalities in organelles as a primary response. Secondary and tertiary alterations, including actin filament depolymerization, disrupted patterns of endocytosis and exocytosis, and cell wall remodeling, ultimately result in perturbed pollen tube extension (Chen et al., 2009). Imaging using fluorescent analogs of CaM reveals a tip-focused gradient, similar to the distribution of cytosolic-free calcium. In long pollen tubes, apical CaM was found to oscillate with a period similar to [Ca\(^{2+}\)]\(_{cyt}\) (Rato et al., 2004).

Reactive oxygen species (ROS) such as the superoxide radical (O\(_2^{-}\)), hydrogen peroxide (H\(_2\)O\(_2\)) and the hydroxyl radical (•OH) have been reported to promote cell expansion by loosening the cell wall (Apel & Hirt, 2004; Schopfer & Liszkay, 2006) and regulating cytoskeleton organization (Fu, 2010). Ca\(^{2+}\)/CaM is involved in plant growth and development by interaction with ROS signaling. For example, activated CaM can either positively or negatively regulate H\(_2\)O\(_2\) concentrations. For positive regulation, Ca\(^{2+}\)/CaM has been proposed to increase H\(_2\)O\(_2\) generation through Ca\(^{2+}\)/CaM-dependent NAD kinase that affects the concentration of available NADPH during assembly and activation of NADPH oxidase (Harding et al., 1997; Lee et al., 1997). For negative regulation, the Ca\(^{2+}\)/CaM complex stimulates the
Cotton fibers (Gossypium hirsutum; cotton lint) are single-celled trichomes that differentiate from the ovule epidermis. Cotton fiber development undergoes several distinct but overlapping steps, including fiber initiation, elongation, secondary cell wall (SCW) biosynthesis and maturation, leading to mature fibers (Tiwari & Wilkins, 1995; Kim & Triplett, 2001). Cotton fiber provides a unique experimental system to study cell elongation and cell wall biosynthesis (Kim & Triplett, 2001), and understanding the molecular control of elongation dynamics offers opportunities for breeding improved fiber quality. Unlike pollen tubes and root hairs, fiber cells elongate via a combination of both tip-growth and diffuse-growth modes, termed the linear cell growth model (Qin & Zhu, 2011). Ca\(^{2+}\) and ROS are two important factors involved in fiber cell tip growth (Qin & Zhu, 2011).

Based on gene expression profile analysis, Ca\(^{2+}\)/CaM is implicated in cotton fiber elongation. Recently, a transcriptome analysis was carried out comparing fiber development in the fiberless (fl) mutant of cotton with that in the wild-type (G. hirsutum cv MCU5) (Padmalatha et al., 2012). Tissues were sampled at fiber initiation (0 d postanthesis, DPA), elongation (5, 10 and 15 DPA) and at the SCW synthesis stage (20 DPA) and analysed using the Affymetrix (Santa Clara, CA, USA) cotton Gene Chip genome array. Data suggest that genes involved in Ca\(^{2+}\) signaling may play a role in fiber cell initiation and differentiation (Padmalatha et al., 2012). Genes associated with CaM and CaM-binding proteins are up-regulated in fiber initials and elongation, also consistent with the view that CaM-mediated signaling could play a role in fiber initiation and elongation (Lee et al., 2007; Taliercio & Boykin, 2007). However, currently, there remains little direct evidence of the mechanism of Ca\(^{2+}\)/CaM on cotton fiber development.

We also cloned a GhCaM gene, with high expression level in elongating fibers, from a normalized cDNA library of sea-island cotton fiber (−2 to 25 DPA; Tu et al., 2007). However, its function in fiber development remains to be illustrated. To further understand the molecular events in Ca\(^{2+}\)-mediated fiber growth, GhCaM7, which had the highest expression level of all the GhCaM gene family members in fiber cells at the fast elongation stage, was overexpressed and down-regulated by RNAi in upland cotton. We show that GhCaM7 is involved in Ca\(^{2+}\) and ROS signal pathways that might play very important roles in fiber development.

Materials and Methods

Plant materials

Cotton (Gossypium hirsutum L.) plants were cultivated in the field at Huazhong Agricultural University, Wuhan, China, according to normal practice. Flowers were tagged on the day of anthesis, and bolls were harvested at 0, 5, 10, 15, 20, 25 d postanthesis (DPA). The fiber cells at different developmental stages were removed carefully from ovules and immediately immersed in liquid nitrogen and stored at −70°C, for further analysis.

Cloning of GhCaM7 cDNA, vector construction and plant transformation

The full-length GhCaM7 (TC232366, 883 bp) and other GhCaM cDNA sequences were obtained from the DFCI Cotton Gene Index (http://compbio.dfc.harvard.edu/cgi-bin/tgi/main.pl?gdb=cotton). The region from 105 to 571 nt containing the coding sequence of the GhCaM7 gene was amplified from 10 DPA fibers by PCR with the proofreading PFu DNA polymerase (Tiangen Biotech, Beijing, China), and then cloned into the pCAMBIA2300 vector (Deng et al., 2012). The 3′ UTR from 609 to 703 nt was amplified and cloned into the RNAi vector (pHellsgate 4). The recombinant plasmids were then introduced into cotton via Agrobacterium-mediated transformation as described by Jin et al. (2006).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated as previously described (Liu et al., 2006), and was reverse-transcribed to cDNA with the SuperScript III reverse transcriptase (Invitrogen). qRT-PCR was performed using the ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA). The primers are listed in the Supporting Information, Table S1. UBQUITIN 7 was used as the housekeeping gene.

Southern, northern and western blots

For Southern blots, genomic DNA was extracted from leaves of cotton plants using a plant genomic DNA kit (Tiangen Biotech). Twenty micrograms of genomic DNA was digested with HindIII, then separated by 0.8% agarose gel electrophoresis and blotted onto Hybond-N+ nylon membranes (Amersham-Pharmacia, Piscataway, NJ, USA) and hybridized with the probe of an NPTII fragment.

For northern blots, 12 μg of RNA was denatured and electrophoresed on a 1.2% agarose gel containing 6% formaldehyde in 1× MOPS (3-(N-morpholino)propanesulfonic acid) buffer. The RNA was then transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia) and hybridized with a GhCaM7 probe fragment, with an rRNA probe as the control.

For western blots, CaM antibody was prepared according to Baum et al. (1996). The proteins collected from the fiber-bearing ovules and leaf were extracted in Laemmli buffer. Gel running, electrophoresis transfer and staining were performed according to Rose et al. (2000). Blots were incubated with primary antibody for 1 h, washed with TBST buffer (Tris-buffered saline with Tween 20) and then incubated with secondary antibody (antimouse immunoglobulin G) for 1 h. Detection was performed using an ECL Western Blotting Substrate Kit (Sigma).

In vitro ovule culture and treatment

Bolls were collected from cotton plants and sterilized in 0.1% (w/v) HgCl\(_2\) for 15 min, followed by three washes with sterile distilled water. The ovules were removed from the bolls under
sterile conditions, and then cultured in BT medium containing H$_2$O$_2$, diphenylcleciodonium (DPI), 2-aminoethoxydiphenylborate (2-APB; Sigma) or added CaCl$_2$ at 30°C in darkness as described by Beasley & Ting (1973). For Ca$^{2+}$ starvation assays, the ovules were cultured at 30°C in darkness in BT medium without Ca$^{2+}$.

**Length measurement of cotton fibers**

Fibers from the field were collected from the bolls from the same positions at the same time. The fiber length was measured manually. To measure the length of the immature (5, 10, and 20 DPA) and *in vitro* cultured fibers, fiber-bearing ovules were first boiled in water to disperse the fibers; these were later clamped with a pincer and flushed in water to straighten them and then dragged onto filter paper and kept at room temperature to dry before measurement. Data were analyzed by Student’s *t*-test.

**Scanning electron microscopy for the observation of fiber initiation**

For the observation of fiber initiation, −1, 0 and 1 DPA ovules were collected from the same positions of cotton plants at c. 18:00 h simultaneously, and fixed in 2.5% (v/v) glutaraldehyde at 4°C. The ovules were dehydrated in an ethanol series (30–100%) at 15 min intervals. Ethanol was replaced by isoamyl acetate/ethanol (1/1) and isoamyl acetate each for 10 min. After critical point drying and ion sputtering coating, the samples were viewed and photographed with a JSM-6390/LV scanning electron microscope.

2',7'-dichlorodihydrofluorescein diacetate (2',7'-DCFDA) staining and imaging

Cotton ovules were detached carefully from bolls and washed with sterile water to remove possible ROS released by cutting. The ovules were then incubated for 30 min in the dark at 30°C in 10 μM 2',7'-DCFDA dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was c. 0.1%. Samples were then washed with sterile water and left for 5 min before imaging. For 0 DPA ovules, fluorescence images were obtained with stereo fluorescence microscopy. For 2 DPA fiber-bearing ovules, a Leica TCS SP2 confocal spectral microscope was used for imaging (Leica, Heidelberg, Germany). Dye excitation was at 488 nm; emitted light was detected at 522 nm. Images were pro-

**2',7'-DCFDA**

- **Length measurement of cotton fibers**
- **Scanning electron microscopy for the observation of fiber initiation**
- **2',7'-DCFDA staining and imaging**
- **Quantification of H$_2$O$_2$ concentrations**

### Results

**GhCaM7 (TC232366) is expressed to high levels in developing fiber cells**

A *GbCaM* gene was isolated from a normalized cDNA library constructed from *Gossypium barbadense* cv 3-79 fibers (Tu *et al.*, 2007). The full length of this *GbCaM* cDNA consisted of 883 nucleotides, encoding a predicted polypeptide of 149 amino acids. This gene showed a high expression level in the fiber elongation phase, consistent with a role for CaM in fiber elongation. Bioinformatics searches of the DFCI Cotton Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=cotton) revealed seven *GbCaM* family members coding four predicted *GbCaM*-isoforms in upland cotton (Fig. 1). To identify *GbCaM* genes that are preferentially expressed in *Gossypium hirsutum* cv YZ1 fibers, the expression patterns of seven genes with *GbCaM* tentative consensus sequences (TCs) were analysed by RT-PCR (Fig. S1). Three members (TC232366, TC244650, and TC256165) were found to be expressed at relatively high levels in fiber cells, especially in the phase of rapid fiber elongation (Fig. S1). Phylogenetic analysis showed that TC232366 and TC244650 had the closest

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evolutionary relationship with the *Arabidopsis* gene *AtCaM7*. TC256165 had the closest evolutionary relationship with the *Arabidopsis* gene *AtCaM6* (Fig. 1a). Alignment of the predicted amino acid sequences of GhCaMs and AtCaMs showed that the four GhCaMs isoforms have very high similarity with AtCaM isoforms (Fig. 1b). qRT-PCR analysis revealed that TC232366 had the highest expression level among these three GhCaM genes; its mRNA abundance at 15 DPA was eight times higher than that of *UBIQUITIN7* (Fig. 2a). TC232366 had the same amino acid sequence as *AtCaM7* (Fig. 1b). Therefore, we termed TC232366 as GhCaM7. Moreover, GhCaM7 and *GbCaM* isolated from the *G. barbadense* 3–79 fiber cDNA library were found to encode the same protein by comparing the predicted amino acid sequences (data not shown).

Various extracellular stimuli elicit specific calcium signatures that can be recognized by different calcium sensors. As one of the most important Ca2+ sensors, CaM can sense an increased Ca2+ influx (Yang & Poovaiah, 2003). Scanning ion-selective electrode analysis showed that the profile of Ca2+ influx at the fiber cell tip was similar to the expression profile of GhCaM7 (Fig. 2b). During fiber elongation, there was an increased Ca2+ influx, whereby the flux rate peaked during plasmodesmatal closure at 10–15 DPA. The rate of Ca2+ influx then decreased during secondary wall synthesis (Fig. 2b).

Several cotton lines with different fiber phenotypes were analyzed phenotypically for fiber initiation by scanning electron microscope (SEM) and a link between phenotype and expression of GhCaM7 was determined using RT-PCR. *G. hirsutum* cv YZ1, Xuzhou 142 and two upland cotton fiber development mutants (Xuzhou 142 *fuzzless lintless*, Xinxiangxiaoji *fuzzless lintless*) were examined in this study. The results presented in Fig. 2(c) show that the sizes of 5 DPA ovules from these cotton lines were very different. The normal upland cotton varieties YZ1 and Xuzhou 142 had normal-sized ovules covered with many fiber cells, whereas the two *fuzzless lintless* mutants had very small ovules with no fiber cells. Moreover, there was no fiber cell initiation from the 0 DPA ovules of the Xuzhou 142 *fuzzless lintless* mutant and the Xinxiangxiaoji *fuzzless lintless* mutant (Fig. 2d). RT-PCR showed that the expression level of GhCaM7 in 0 DPA ovules was lower in the *fuzzless lintless* mutants than in the normal upland cotton lines YZ1 and Xuzhou 142 (Fig. 2e). The results are consistent with a role for GhCaM7 in fiber initiation and elongation.

Fig. 1 Phylogenetic relationships between *Gossypium hirsutum* calmodulin 7 (GhCaM7; TC232366) and other reported calmodulin (CaM) proteins from *Arabidopsis thaliana*. (a) The four CaM isoforms encoded by the seven GhCaMs (*G. hirsutum* calmodulins) are aligned with *Arabidopsis* CaMs. The regions corresponding to the E helices, Ca2+-binding loops and F helices are indicated by black, calamine blue and black bars, respectively. (b) Phylogenetic relationships of *G. hirsutum* and *A. thaliana* CaMs. AtCaM1, at5g37780; AtCaM2, at2g41110; AtCaM3, at3g56800; AtCaM4, at1g66410; AtCaM5, at2g27030; AtCaM7, at3g43810.
Overexpression of GhCaM7 promotes early but not late elongation of fiber cells

To examine the effects of GhCaM7 on fiber development, RNAi and overexpression vectors of GhCaM7 were constructed and used to transform the upland cotton variety YZ1. Eight independent transgenic GhCaM7 RNAi cotton lines and six independent GhCaM7 overexpression cotton lines (T0) were obtained. After analysis by Southern blots, qRT-PCR, northern blots and western blots, two RNAi lines (i28 and i27) and two overexpression lines (O11 and O14), each with one T-DNA copy insertion, were identified for further analysis (Figs 3, S2).

Quantitative RT-PCR and northern blotting showed that the transcript abundance of GhCaM7 in the RNAi lines was significantly reduced, and that in 5 DPA fibers of overexpression lines was significantly increased, compared with the wild-type (Fig. 3). At 10 DPA, GhCaM7 was overexpressed to a small extent (≥1.5 times) in the fibers of overexpression lines compared with the wild-type. The transcript abundance of GhCaM7 in the RNAi lines was significantly reduced in 10 DPA fibers compared with the wild-type (Fig. 3). These results were confirmed for GhCaM7 protein levels by western blotting (Fig. S2b).

Fiber length, which is the key determinant of fiber quality and output, was compared between the transgenic lines and the wild-type. The lengths of fibers in RNAi lines (T2 plants) were shorter than in the wild-type (28.3 ± 1.7 and 28.5 ± 1.9 mm for lines i28 and i27, respectively, i.e. 9.6% and 8.9% shorter than the wildtype, 31.3 ± 0.8 mm). This result was also seen in T3 RNAi plants (25.9 ± 2.9 and 25.9 ± 1.5 mm, respectively, compared with the wild-type, 28.7 ± 1.1 mm; Fig. S3). T2 and T3 plant overexpression lines showed no statistically significant difference in fiber length compared with the wild-type (Fig. S3).

Overexpression vectors of GhCaM7 were used to transform the upland cotton variety YZ1. Eight independent transgenic GhCaM7 RNAi family members preferentially expressed in fibers. Total RNA samples were extracted from various developmental stages and were used for qRT-PCR analysis. Cotton (G. hirsutum) UBQ7 (GenBank accession no. DQ116441) was included as the template control. The qRT-PCR results were obtained from three independent RNA extractions. Error bars, ± SD of four technical repeats.

(b) Ca²⁺ flux during fiber development was measured by a noninvasive scanning ion-selective electrode technique. During fiber elongation, there was an increasing Ca²⁺ influx from the extracellular to the intracellular environment and the flux rate peaked during plasmodesmatal closure from 10 to 15 d postanthesis (DPA). The rate of Ca²⁺ influx subsequently decreased during the secondary cell wall synthesis. The inset (lower left) is a schematic diagram for the measurement of Ca²⁺ flux in testing buffer by noninvasive scanning ion-selective electrode. Error bars, ± SD of nine samples. (c–e) Morphological and GhCaM7 expression analysis in wild-type and fiber mutant lines. (c) Ovules at 5 DPA from Xuzhou 142 fuzzless/lintless mutants fl and J fuzzless/ lintless mutants fl do not have fibers compared with the wild-type genotypes YZ-1 and Xuzhou 142. Bars, 10 mm. (d) Scanning electron micrograph images showing that the Xuzhou 142 fuzzless/lintless mutants fl and J fuzzless/lintless mutants fl have no fiber initials on the surface of 0 DPA ovules, but both the wild-type genotypes show rich fiber initials. (e) Reverse transcription polymerase chain reaction (RT-PCR) shows that the expression levels of GhCaM7 are correlated with the fiber initiation status. For 0 DPA ovules, bars = 200 μm; for fiber initiation images taken at a similar position in the middle of ovules, bars = 50 μm. For RT-PCR analysis, cotton UBQ7 was used as the internal control to normalize expression levels.
10.5 ± 0.8 and 8.6 ± 0.6 mm, respectively, significantly longer than the wild-type fiber cells (7.6 ± 0.5 mm). However, fiber elongation was inhibited significantly in GhCaM7 RNAi lines i28 and i27 (Fig. S4a). Moreover, pharmacological experiments revealed that fiber elongation was inhibited by the CaM antagonist TFP in a dose-dependent manner. When the 5 μM TFP concentration was applied, the average fiber length was 6 mm (i.e., about half that of the control). When the applied TFP concentration was increased to 20 μM, fiber elongation was severely inhibited and the length only reached c. 1 mm (Fig. S4b). These data indicate a role for GhCaM7 in regulating early fiber elongation.

GhCaM7 is a positive regulator of ROS in cotton early fiber elongation

To investigate whether ROS influences cotton fiber development, 0 DPA ovules were collected for ovule culture assays and treated with H2O2 for 8 d. Treatment with 2, 5, 10 and 20 μM H2O2 led to significantly increased fiber length compared with untreated controls, while higher concentrations (up to 50 μM H2O2) led to inhibition of fiber growth (Fig. 5a). Ovule culture assay and H2O2 quantitative analysis showed that Ca2+ starvation promoted H2O2 production in ovules and early fiber elongation (Figs 5b, S5). Moreover, 2′,7′-DCFDA staining and imaging of fiber-bearing ovules indicated that exogenous Ca2+ starvation could induce the production of ROS (Fig. 5d), consistent with the observed promotion of fiber elongation. Additional treatment with 20 μM H2O2 increased the ROS concentrations and the extent of early fiber elongation in conditions of Ca2+ starvation (Fig. 5c,d); while addition of the ROS inhibitor DPI (1 μM) blocked fiber elongation completely (Fig. 5c). These results show the positive role of ROS on early fiber elongation.

Given that Ca2+/CaM can be involved in plant growth and development by interacting with ROS signaling (Harding et al., 1997), we investigated whether GhCaM7 was involved in fiber elongation by regulating ROS production. ROS concentrations were determined in fiber-bearing ovules of wild-type and transgenic lines, using 2′,7′-DCFDA staining and imaging. The results showed that ROS concentrations were increased in ovules of GhCaM7 overexpressers at 0 and 2 DPA, and were reduced in 0 and 2 DPA RNAi lines, compared with the wild-type (Fig. 6a,b). H2O2 quantification showed that at 2 and 5 DPA, GhCaM7-overexpression ovules and fibers had significantly higher concentrations of H2O2 than wild-type or RNAi lines (Fig. 6c,d). At 10 DPA, GhCaM7-overexpression fibers had slightly higher concentrations of H2O2 and GhCaM7-RNAi fibers had lower concentrations of H2O2 than the wild-type (Fig. 6e).

An ovule culture assay was then performed to further analyze the relationship between GhCaM7 and ROS. As shown in Fig. S6(a), 10 μM H2O2 relieved the inhibitory effects on fiber elongation in GhCaM7 RNAi lines. When treated with 50 μM H2O2 for 10 d, the cultured ovules of overexpression and wild-type plants developed shorter fibers than controls, and the effect was greater for overexpressers (Fig. S6b). This suggests that overexpression of GhCaM7 leads to enhanced sensitivity to H2O2. Consistent with this, RNAi lines showed resistance to the growth inhibitory effects of 50 μM H2O2. Moreover,
transgenic and wild-type ovules were collected and cultured in BT medium in the presence of 0.5 μM DPI for 8 d. In this condition there was no significant difference in fiber length among ovules of overexpressers, RNAi lines and the wild-type (Fig. S6c).

Furthermore, TFP (a CaM antagonist) treatment of developing fibers was used to investigate the link between CaM and ROS. It was found that TFP inhibited ROS production as well as fiber production (Fig. 6f–h). Significantly, when 20 μM H₂O₂ was applied together with TFP, ROS concentrations in fiber cells were elevated and the inhibitory effect of TFP on fiber elongation was partly relieved (Fig. 6g–h). This supports the view that ROS production is downstream of CaM and required for fiber elongation. When 1 μM of the ROS inhibitor DPI was applied, ROS concentrations in the fiber cells were suppressed and fiber cell development was inhibited (Fig. 6i–l). Therefore, TFP treatment appears to block the production of ROS to inhibit fiber elongation and so GhCaM7 is likely to act upstream of ROS production.

ROS might feedback-regulate GhCaM7 levels by increasing [Ca²⁺]_{cyt}

We further examined the relationship between GhCaM7 expression and calcium starvation. qRT-PCR analysis showed that the expression of GhCaM7 was induced by exogenous Ca²⁺ starvation (Fig. 7a), consistent with a role for GhCaM7 as a positive regulator of fiber elongation. According to the conclusion that GhCaM7 can act upstream of ROS production, we speculated that the ROS induced by exogenous Ca²⁺ starvation as described in Fig. 5(b) and (d) might be the result of the increased expression level of GhCaM7. However, unexpectedly, H₂O₂ quantification showed that TFP could not inhibit the production of ROS induced by exogenous Ca²⁺ starvation (Fig. 7b). We conclude that ROS and GhCaM7 have a different relationship under conditions of exogenous Ca²⁺ starvation.

Then, by qRT-PCR, we found that the expression of GhCaM7 was induced by exogenous 20 μM H₂O₂ under both the normal condition and the treatment with exogenous Ca²⁺ starvation (Fig. 7c). Treatments with 1 μM DPI down-regulated the expression of GhCaM7 (Fig. 7c). It is recognized that ROS modulates Ca²⁺ homeostasis by activating Ca²⁺ channels and depressing Ca²⁺ pumps (Yan et al., 2006). We speculated that ROS might feedback-regulate GhCaM7 levels by increasing [Ca²⁺]_{cyt}.

In order to confirm this possibility, scanning ion-selective electrode analysis was performed and the results showed that when treated with 20 μM H₂O₂, there was an increasing rate of Ca²⁺ influx at the fiber cell tip (Fig. 7f). Moreover, ovule culture assay experiments showed that when 0 DPA ovules were cultured in BT medium without Ca²⁺ for 15 d, the ovules underwent necrosis, while addition of 20 μM H₂O₂ prevented necrosis (Fig. 7d). One explanation for this is that
H$_2$O$_2$ might promote Ca$^{2+}$ release from intracellular calcium pools. In order to test this possibility, a calcium pool release channel blocker 2-APB (Bootsman et al., 2002) was used in ovule culture. As shown in Fig. 7(a) and (e), when there was no exogenous calcium, H$_2$O$_2$ could promote the expression level of $GhCaM7$ and fiber elongation, while in the presence of 2-APB, H$_2$O$_2$ had a negligible effect on the expression level of $GhCaM7$ and fiber elongation. From these results, we concluded that H$_2$O$_2$ could trigger the Ca$^{2+}$ signal by increasing Ca$^{2+}$ influx from the extracellular environment, or promoting Ca$^{2+}$ release from intracellular calcium pools. We speculate that this might lead to the Ca$^{2+}$ signal being sensed by $GhCaM7$ and the expression level of $GhCaM7$ being up-regulated.

Moreover, qRT-PCR showed that, in $GhCaM7$ overexpression 1 DPA fibers with greater ROS accumulation, two Ca$^{2+}$ channel genes, CNGC-1 and -2, were up-regulated and two Ca$^{2+}$ pump genes, encoding Ca$^{2+}$-ATPase-1 and -5, were down-regulated compared with the wild-type. In $GhCaM7$ RNAi 1 DPA fibers that were defective in ROS production, both Ca$^{2+}$ pump genes were up-regulated, although both Ca$^{2+}$ channel genes were unchanged (Fig. 7g–h). These data are consistent with a model whereby ROS regulates $GhCaM7$ expression through positive feedback by increasing [Ca$^{2+}$]$_{cyt}$.

**Discussion**

Cotton fiber elongation is initiated largely by cell wall loosening and terminated by increased wall rigidity and loss of turgor (Ruan et al., 2001). Ca$^{2+}$ and ROS are reported to play important roles in the early stages of fiber elongation, mainly on the basis of evidence from transcription analysis (Lee et al., 2007; Taliercio & Boykin, 2007; Mei et al., 2009; Zhang et al., 2010; Padmalatha et al., 2012; Walford et al., 2012). Any interactions between the two factors during fiber elongation remain unexplored. Here, we report that a Ca$^{2+}$ sensor, $GhCaM7$, is preferentially expressed in elongating fibers (Figs 2a, S1). Using transgenic and cell biological approaches, we provide evidence that GhCaM7, Ca$^{2+}$ and ROS are three important regulators involved in early fiber development.

Calcium signals represent a core regulatory system in plant cell physiology and cellular responses to the environment (Dodd et al., 2010). Tip-focused Ca$^{2+}$ gradients are important determinants of polarity in tip-growing cells such as root hairs, pollen tubes, fungal hyphae and algal rhizoids (Hepler et al., 2001). Ca$^{2+}$ influx is important for maintaining tip-focused Ca$^{2+}$ gradients. During fiber development, there is a sustaining Ca$^{2+}$ influx at the tip of fiber cells and the flux rate peaks during plasmodesmatal closure at 10–15 DPA (Fig. 2b), corresponding to
the observation that a high Ca\(^{2+}\) gradient is found in the cytoplasm of rapidly elongating cotton fiber cells near the growing tip (Huang et al., 2008). When the Ca\(^{2+}\) signal was disrupted by the Ca\(^{2+}\) pool release channel blocker 2-APB, fiber elongation was inhibited (Fig. S7), supporting a role for Ca\(^{2+}\) in fiber development.

As one of the most important Ca\(^{2+}\) sensors, CaMs are essential for plant cell elongation, such as in the pollen tube and cotton fiber. Pollen tube and cotton elongation can be inhibited by extracellular application of CaM antagonists (Picton & Steer, 1985; Rato et al., 2004; Chen et al., 2009). Down-regulating GhCaM7 leads to shorter cotton fibers, whereas overexpressing GhCaM7 promotes early fiber elongation (Fig. 4). We showed that GhCaM7 is involved in cotton fiber elongation by modulating ROS production, which also plays a positive role in cotton fiber cell initiation and early elongation. 2',7'-DCFDA staining of ROS production showed that extremely strong fluorescent signals are present in 0–2 DPA fiber cells, whereas almost no signal was detected in −1 DPA wild-type or −1 to 2 DPA fuzzless/limitless fl (mutant) ovules. ROS was generated to a significant degree during cotton fiber initiation. In vivo and in vitro treatments of ovules demonstrated that 30% H\(_{2}\)O\(_{2}\) can prevent retardation of fiber initiation in Xinxiangxiao Linted-Fuzzless Mutants (XinFLM). The expression of the fiber initiation-related gene GhMYB25 increased significantly after 30% H\(_{2}\)O\(_{2}\) treatment for 24 h (Machado et al., 2009; Zhang et al., 2010). Treatment with H\(_{2}\)O\(_{2}\) < 20 lM led to significantly increased fiber length (Fig. 5a). It has been suggested that ROS may function in early fiber elongation through the ethylene pathway or by affecting the fiber cell wall directly (Qin & Zhu, 2011).

Calcium/CaM has been proposed to increase H\(_{2}\)O\(_{2}\) generation through a Ca\(^{2+}/CaM\)-dependent NAD kinase that affects the concentration of available NADPH during assembly and activation of NADPH oxidase (Harding et al., 1997; Lee et al., 1997). NADPH oxidase plays a central role in the oxidative burst. Arabidopsis thaliana NADPH oxidase mutants are defective in root hair elongation (Foreman et al., 2003). NADPH oxidase is also crucial for cotton fiber development. Blocking the activity of the NADPH oxidase with DPI inhibits ROS formation and fiber elongation.

![Fig. 6](image_url) GhCaM7 (Gossypium hirsutum calmodulin 7) regulates reactive oxygen species (ROS) production. (a) Fluorescence and bright field image of ROS in 0 d postanthesis (DPA) ovules of transgenic and wild-type lines (bar, 1 mm). The images were taken at a 0.25 s exposure. (b) Fluorescence and bright field image of ROS in 2 DPA fibers of transgenic and wild-type fiber lines. Bars, 150 µm. (c) Hydrogen peroxide (H\(_{2}\)O\(_{2}\)) quantification of transgenic and wild-type lines in 2 DPA fiber-bearing ovules. ***, P < 0.01. Error bars, ±SD calculated from six cotton bolls. (d, e) H\(_{2}\)O\(_{2}\) quantification of transgenic and wild-type lines in 5 and 10 DPA fibers. ***, P < 0.01. Error bars, ±SD calculated from four cotton bolls. (f) H\(_{2}\)O\(_{2}\) quantification shows that trifluoperazine (TFP, 10 µM) and diphenyleneiodonium (DPI, 1 µM) inhibit H\(_{2}\)O\(_{2}\) production in fibers cultured in vitro for 10 d. ***, P < 0.01. Error bars, ±SD calculated from four cotton bolls. (g) H\(_{2}\)O\(_{2}\) partly relieves the inhibitory effect of TFP on fiber elongation (bar, 10 mm). The SD was calculated from at least 20 ovules. (h) Fluorescence and bright field image of ROS in 1 DPA fiber. TFP (10 µM) and DPI (1 µM) inhibits ROS production and exogenous H\(_{2}\)O\(_{2}\) partly relieves the inhibitory effect of TFP on ROS production. Bars, 100 µm. WT, wild-type; O11, O14, GhCaM7 overexpression lines; i28, i27, GhCaM7 RNAi lines.

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cell elongation (Figs 5c, 6f–h). DPI (0.5 μM) can abolish the significant difference in fiber lengths among ovules of GhCaM7 overexpresser, RNAi and wild-type plants (Fig. 5c). Therefore, we infer that GhCaM7 might modulate ROS production by activating NADPH oxidase. However, more evidence is needed to support this conclusion.

Calcium signaling and ROS may generate a positive feedback loop to maintain plant cell elongation (Takeda et al., 2008). It is proposed that during the development of root hairs, ROS derived from the RHD2 NADPH oxidase at the tip may activate hyperpolarization-activated Ca^{2+} channels that transport Ca^{2+} into the cells, which in turn activates RHD2 NADPH oxidase activity through its EF hand and a Ca^{2+}-dependent protein kinase activity. Such a system of positive feedback, in concert with an independent mechanism for locating RHD2 protein at the tip of the cell, provides a robust mechanism that can explain how cells such as root hairs maintain polarity during morphogenesis (Takeda et al., 2008). We propose that a similar loop might exist in cotton fiber development. During fiber elongation, Ca^{2+}/GhCaM7 regulates ROS production. ROS may release Ca^{2+} from the Ca^{2+} pool (Pariente et al., 2001; Fig. 7d,e). GhCaM7 is activated by the increased [Ca^{2+}]_{cyt}, and we suggest it may function by making more ROS by targeting NAD kinase (Harding et al., 1997). Depending on the concentration, H_{2}O_{2} can induce cell protective responses, programmed cell death, or necrosis (Levine et al., 1994). Although ROS can function as a positive factor in fiber elongation, a high concentration of ROS will inhibit fiber elongation (Fig. 5a). During fiber development, there is a high concentration of ROS in fiber cells at the stage of secondary wall formation. H_{2}O_{2} can function as a developmental signal in the differentiation of secondary walls in cotton fibers. Exogenous addition of H_{2}O_{2} prematurely promotes secondary wall formation in young fibers (Potikha, 1999), and early secondary wall formation reduces fiber elongation. This process may explain our observation that GhCaM7 overexpression plants had slightly shorter mature fibers (Figs 4b, S3).

In summary, we show that GhCaM7 plays a critical role in fiber cell initiation and elongation by modulating ROS production, and we provide a molecular link between Ca^{2+} and ROS signal pathways in fiber development.
Acknowledgements
The technical assistance from Lihong Qin (Huazhong Agricultural University, China) for SEM and Hillel Fromm (Department of Molecular Biology and Ecology of Plants, Israel) for western blots antibodies was greatly appreciated. This work was financially supported by the National Natural Science Foundation of China (no. 31230056).

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Supporting Information
Additional supporting information may be found in the online version of this article.

Fig. S1 Tissue expression patterns of seven CaM family members by RT-PCR.

Fig. S2 Southern and western blotting analysis of GhCaM7 in transformed cotton plants.

Fig. S3 Two years of fiber length statistics of GhCaM7 transgenic and wild-type lines.

Fig. S4 In vitro validation of the role of GhCaM7 in fiber elongation by ovule culture assay.

Fig. S5 Ca2+ starvation promotes the early elongation of cotton fibers.

Fig. S6 Effects of H2O2, DPI and Ca2+ starvation treatments on overexpression, RNAi and wild-type cultured ovules.

Fig. S7 Effect of 6 d of treatment with different concentrations of 2-APB on fiber and ovule development.

Table S1 Primers used in this study

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