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Down-regulating annexin gene *GhAnn2* inhibits cotton fiber elongation and decreases Ca^{2+} influx at the cell apex

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Abstract Cotton fiber is a single cell that differentiates from the ovule epidermis and undergoes synchronous elongation with high secretion and growth rate. Apart from economic importance, cotton fiber provides an excellent single-celled model for studying mechanisms of cell-growth. Annexins are Ca^{2+}- and phospholipid-binding proteins that have been reported to be localized in multiple cellular compartments and involved in control of vesicle secretions. Although several annexins have been found to be highly expressed in elongating cotton fibers, their functional roles in fiber development remain unknown. Here, 14 annexin family members were identified from the fully sequenced diploid *G. raimondii* (D_5 genome), half of which were expressed in fibers of the cultivated tetraploid species *G. hirsutum* (cv. YZ1). Among them, *GhAnn2* from the D genome of the tetraploid species displayed high expression level in elongating fiber. The expression of *GhAnn2* could be induced by some phytohormones that play important roles in fiber elongation, such as IAA and GA_3. RNAi-mediated down-regulation of *GhAnn2* inhibited fiber elongation and secondary cell wall synthesis, resulting in shorter and thinner mature fibers in the transgenic plants. Measurement with non-invasive scanning ion-selective electrode revealed that the rate of Ca^{2+} influx from extracellular to intracellular was decreased at the fiber cell apex of *GhAnn2* silencing lines, in comparison to that in the wild type. These results indicate that GhAnn2 may regulate fiber development through modulating Ca^{2+} fluxes and signaling.

Keywords *Gossypium hirsutum* (cotton) · GhAnn2 · Fiber elongation · Fiber secondary cell wall synthesis · Calcium signaling

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Introduction

Annexins are Ca^{2+}- and phospholipid-binding proteins with a broad taxonomic distribution covering prokaryotes, fungi, protists, plants and higher vertebrates (Gerke and Moss 2002; Morgan et al. 2004, 2006). The overall structure of annexins is evolutionary conserved. The C-terminal core of annexin consists of four annexin repeats and each repeat comprises five short α-helices (Laohavisit

and Davies 2011). In the animal annexin, each of the four repeats contains a conserved endonexin fold (K-G-X-G-T-{38}-D/E) that can bind a single Ca^{2+} ion (Kourie and Wood 2000; Moss and Morgan 2004). Calcium enables the reversible binding of annexins to negatively charged phospholipids, and the Ca^{2+} requirement for binding can be reduced by acidic pH (Blackbourn et al. 1991). Differing from their animal counterparts, only the first and fourth repeated domains of plant annexins have the characteristic endonexin sequence. In addition, plant annexins have larger surface area due to extra grooves and clefts in comparison to mammalian annexins, suggesting that plant annexins may have wider range of interaction partners and a broad range of roles within the cell (Mortimer et al. 2008).

Plant annexins are expressed throughout the life cycle of a plant and are regulated by developmental and environmental signals. However, the functions of these proteins remain poorly understood. Most of what is described about plant annexin functions comes from in vitro studies. These include their involvement in exocytosis, actin binding, peroxidase activity, callose synthesis and ion transport (Laohavisit and Davies 2011). The expression of annexin has been found to be regulated by plant hormones such as abscisic acid (Xin et al. 2005), gibberellic acid (Lu et al. 2012), jasmonic acid (Kiba et al. 2005), auxin (Baucher et al. 2011), and salicylic acid (Gidrol et al. 1996; Konopka-Postupolska et al. 2009). Plant annexin genes can be induced by biotic stresses such as a pathogen attack (de Carvalho-Niebel et al. 2002), as well as by abiotic stresses including salt, drought, wounding, heat or cold, heavy metal and oxidative stresses (Cantero et al. 2006; Konopka-Postupolska et al. 2009; Divya et al. 2010; Jami et al. 2011). Of particular interest is that plant annexins are usually prominent at the apex of cells undergoing polar elongation, such as root hairs, pollen tubes and fern rhizoids (Mortimer et al. 2008), suggesting that plant annexins are also involved in cell elongation.

Cotton fibers (cotton lint) are single cells differentiated from the ovule epidermis that undergo rapid and synchronous elongation, which serve as the mainstay of the modern textile industry. Because of its unicellular and linear structures, cotton fiber is an ideal model for studies of plant cell elongation and cell wall biosynthesis (Kim and Triplett 2001). Cotton fiber development includes five overlapping stages: initiation, elongation, transitional wall thickening and primary wall remodeling, secondary wall synthesis and maturation (Haigler et al. 2012). The mature fiber can reach to 30–50 mm in length. It is hypothesized that cotton fiber may elongate via linear cell-growth mode, a combination of both tip-growth and diffuse-growth modes (Qin and Zhu 2011). Tip-growth is important for cell elongation, tip-localized Ca^{2+} gradient and active secretary vesicle trafficking are two important aspects of this mode of growth (Cole and Fowler 2006). Thus, genes such as annexins that associate with Ca^{2+} and active secretary vesicle trafficking will be good candidates for studying the mechanism of fiber cell elongation.

Early in vitro activity assay showed that cotton fiber annexins could inhibit β-glucansynthase activity (Andrawis et al. 1993). However, a recombinant cotton annexin expressed in *Escherichia coli* did not inhibit β-glucansynthase activity, instead it was capable of hydrolyzing ATP and GTP. The GTPase activity of this annexin was much greater than its ATPase activity. Northern-blot analysis showed that the annexin gene was highly expressed in the elongation stages of cotton fiber differentiation, suggesting a role of this annexin in cell elongation (Shin and Brown 1999). The ligon mutant of cotton (*Gossypium hirsutum*) is impaired in fiber elongation, and proteomic analysis revealed significant downregulation of five annexin isoforms compared to the wild type, also suggesting annexins may play roles in fiber development (Zhao et al. 2009). In the recent years, heterologous expression of cotton fiber annexins provided some cues for their molecular function in cell development. For example, GhAnx1, which could bind Ca^{2+} in vitro, was isolated from a cotton (*G. hirsutum* cv. CRI35) fiber cDNA library. *E. coli* cells expressing GhAnx1 were protected from tert-butyl hydroperoxide (tBH) stress, suggesting that it had a potential antioxidative role (Zhou et al. 2011). Overexpression of *AnnGh3* in *Arabidopsis* resulted in a significant increase in trichome density and length on leaves of the transgenic plants, suggesting that AnnGh3 may be involved in fiber cell initiation and elongation of cotton (Li et al. 2013a, b). In addition, *AnxGb6* is a *G. barbadense annexin* gene that was found to be specifically expressed in fiber. Yeast two hybridization and BiFC analysis revealed that a AnxGb6 homodimer interacted with a cotton fiber specific actin GbAct1. And ectopic-expression of *AnxGb6* in *Arabidopsis* enhanced root elongation and resulted in more F-actin accumulation in the basal part of the root cell elongation zone. Therefore, it was suggested that *AnxGb6* may be important in fiber elongation by potentially providing a domain for F-actin organization (Huang et al. 2013).

Despite the progress described above, the *in planta* role of annexins in fiber elongation still remains unclear and the answer to this relies on functional analyses in transgenic cotton. To achieve this goal, we identified 14 annexin family members from the fully sequenced *G. raimondii* (D_5 genome), half of which were expressed in fibers of cultivated *G. hirsutum* (cv. YZ1). One of them, *GhAnn2* was highly expressed in elongating fibers and was thus chosen for functional analyses by using a transgenic approach. The data show that RNAi silencing of *GhAnn2* decreased the Ca^{2+} influx at the tips of fiber cells and reduced fiber elongation and cell wall thickness. We conclude that GhAnn2

may control fiber development through regulating Ca^{2+} fluxes and signaling.

Materials and methods

Plant materials

Cotton *G. hirsutum* cv. YZ1 was used in this study. The plants were cultivated in the experimental field of Huazhong Agricultural University (Wuhan, China). Ovules and fibers were removed carefully from developing flower buds or bolls on selected days post anthesis (DPA). Petal, stigma and anther were separated from the flower in the field at day of anthesis. Roots, stems and leaves were collected from 21-day-old seedlings. All the samples were frozen immediately and stored at −70 °C before use.

Sequence analysis of annexin family members in *Gossypium raimondii* (D genome cotton)

Using annexin as keywords, annexin family members in *Gossypium raimondii* (D genome) and *Arabidopsis* were searched in the Phytozome 9.1 database (http://www.phytozome.net/). Phylogenetic analysis was performed with ClustalX program (version 1.83) (Thompson et al. 1997) and MEGA4 (Tamura et al. 2007) by the Neighbor-Joining method.

Gene cloning, sequence analysis, vector construction and transformation

Based on an EST sequence of *GbAnn* isolated from a normalized cDNA library of *G. barbadense* cv. 3–79 elongating fiber at the elongation stage (Tu et al. 2007), *GhAnn2* was obtained by PCR amplification using 10 DPA cotton fiber cDNA of *G. hirsutum* cv. YZ1 as the template. Gene-specific primers are listed in Supplementary Table S7. In order to determine which subgenome *GhAnn2* comes from, cotton annexin EST were obtained from the database of NCBI (http://www.ncbi.nlm.nih.gov/nucest/) and assembled by CAP3 (Huang and Madan 1999). Tablet (http://bioinf.scri.ac.uk/tablet/) was used to visualize the SNPs by sequence assemblies and alignments.

For the RNAi vector construction, a pair of primers with attB1 and attB2 adaptors was designed at the 1014th and 1124th nucleotides of the *GhAnn2* sequence (Supplementary Table S7). The PCR product was cloned into pHellsgate4 (Helliwell et al. 2002) according to the manufacturer's recommendations and then the constructed vectors were introduced into *G. hirsutum* YZ1 plants by *A. tumefaciens* using strains LBA4404 (Jin et al. 2006).

qRT-PCR, Southern and Northern bolts

Total RNA was extracted as previously described (Liu et al. 2006). For qRT-PCR, 3 μg RNA was reverse-transcribed to cDNA with the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Then qRT-PCR was performed using the ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). For Northern blotting, 12 μg RNA was denatured and electrophoresed on a 1.2 % agarose gel containing 6 % formaldehyde in 1× MOPS buffer. The RNA was then transferred to a Hybond-N^+ nylon membrane (Amersham-Pharmacia, USA) and hybridized with a *GhAnn2* probe fragment labeled by P^{32}, with rRNA as the control.

Genomic DNA for Southern blots was extracted from leaves of cotton plants by the CTAB method with a plant genomic DNA kit DP305 (Tiangen Biotech, Beijing). Twenty micrograms genomic DNA was digested with *Hind*III, then separated by 0.8 % agarose gel electrophoresis and blotted onto Hybond-N^+ nylon membranes (Amersham-Pharmacia, USA) and hybridized with the probe of *NPTII* fragment labeled by P^{32}.

Ovule culture and La^{3+} treatment

Bolls were collected from cotton plants at 0 DPA (about 6:00 pm) and sterilized in 0.1 % (w/v) $HgCl_2$ for about 15 min. After three washes with sterile distilled water, the ovules were removed from the bolls under sterile conditions, floated on liquid BT medium (0.5 μM GA_3, 5 μM IAA) in a flask, and cultured in the dark at 30 °C (Beasley and Ting 1973). For La^{3+} treatment (Yuasa et al. 1998), the ovules from the RNAi lines and the wild-type were cultured in BT medium containing 7 or 28 μM $LaCl_3$ (Sigma) for 10 days. More than 10 cultured ovules per treatment were used for fiber length measurement.

Length and quality measurements of cotton fiber

Fibers from the field were collected from the bolls at the same positions of the plants at the same time. The length of mature fiber was measured with a ruler after making the fiber straight by using a comb. For quality assessment of cotton fiber, more than 7 g fiber was sent to the Center for Cotton Fiber Quality Inspection and Testing at the Chinese Ministry of Agriculture (Anyang, Henan, China). Three biological replicates were performed. Data were analyzed using Student's *t* test. For length measurement of the immature fibers (5, 10, 15 DPA) and in vitro cultured fibers, fiber bearing ovules were first boiled in the water to make the fibers dispersed and then clamped with forceps and flushed under the water tap to make the fiber straight, and then the fibers were dragged on the filter paper, and then kept at room temperature to dry for measurement.

Cotton fiber paraffin section

Fibers on the seeds were combed to make sure the fiber cells are straight. A bunch of fiber cells at the same position of the cotton seed were bundled with a fine thread and then stripped from the seed. Tissues were fixed with FAA for 24 h at room temperature, dehydrated with ethanol and trichloromethane (Wang et al. 2009). After paraffin bathing, tissues were embedded in paraffin blocks for sectioning (8 μm). Sections were dewaxed with xylene for 1 h, which was repeated for 0.5 h, washed for 10 s each in 100 % ethanol, 95 % ethanol, 85 % ethanol, 70 % ethanol, 50 % ethanol, 30 % ethanol and distilled water. Sections were stained with safranine (1 %) for 5 min, and then washed for 10 s with distilled water, 30 % ethanol, 50 % ethanol, 70 % ethanol and 85 % ethanol, then stained with Fast green (FCF, 0.5 %) for 20–25 s, washed for 10 s with 95 % ethanol, 100 % ethanol, 50 % ethanol + 50 % xylene, then placed in 100 % xylene. Finally, the samples were observed under the light microscope. The thickness of the fiber cell wall was measured with the software of Image-Pro Plus 6.0 (Media Cybernetics, Inc., USA). About 50 fiber cells from six seeds were measured and data were analyzed by the Student's *t* test.

Measurement of extracellular Ca^{2+} influx

Fresh 5 DPA bolls of the RNAi lines and the wild-type were collected at the same position on the plant and the same time. Fiber bearing ovules in one chamber of the boll were carefully removed and equilibrated in 5 ml testing liquid (0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, 0.1 % sucrose, pH 6.0) for 0.5 h. Then Ca^{2+} flux of the fiber cell apex was measured in another 5 ml testing liquid by Xu-Yue Science and Technology Co. (www.xuyue.net) using the non-invasive, scanning ion-selective electrode technique as described previously (Shabala et al. 1997). Four bolls in each line were analyzed, and at least three fiber cells per boll were tested. The generated data of potential difference was used for the calculation of Ca^{2+} flux. The average rates of influx were plotted on the graph.

Results

GhAnn2 is preferentially expressed in fast elongating cotton fibers

When we analyzed the gene expression profiles of *G. barbadense* cv. 3–79 fibers, an annexin gene expressed preferentially during elongation stage was isolated (Tu et al. 2007, KC316004.1). Due to technical difficulties in transforming *G. barbadense*, we identified its homolog from the transformable *G. hirsutum* for determining its in vivo function. Based on primes for the sequence of this *Gb* annexin, we isolated a full-length cDNA clone consisting of 1,269 nucleotides, encoding a polypeptide of 316 amino acids (Fig. S1). This protein, named GhAnn2, shared 99 % identity with the previously identified, *Gb* annexin (Tu et al. 2007). Its predicted amino acid sequence contained a typical annexin domain, which consisted of four repeats (Repeat 1–4). Repeat 1 contained a type-II Ca^{2+}-binding site (G-X-G-T-{38}-E). Trp27 (W) and His40 (H) in Repeat 1 is important for phospholipid-binding (Delmer and Potikha 1997) and peroxidase activity of annexins (Mortimer et al. 2008), respectively. IRI in Repeat 3 was an F-actin-binding site, and in Repeat 4, there was a GTP-binding site (position 298–301 DXXG; Fig. S1). Multiple sequence alignment analysis showed the predicted amino acid sequence of this annexin had only two amino acids difference with AnnGh2 (AAB67994; Potikha and Delmer 1997). Moreover, the nucleotide sequence of ORF in this annexin had 99 % identity with *AnnGh2* (Table S4). As a result, this annexin was named *GhAnn2* according to the gene nomenclature conventions.

Annexins are highly expressed in cotton fiber, have therefore drawn attention from many researchers (Shin and Brown 1999; Tu et al. 2007; Wang et al. 2010a, b; Zhou et al. 2011; Huang et al. 2013; Li et al. 2013a). And as a result, many annexins have been cloned and named. In order to rationalize the relationship among these annexins, we attempted to identify all the annexins in cotton. Firstly, by searching the database of Phytozome v 9.1 (http://phytozome.net/) using annexin as a keyword, 14 annexin family members were identified in *G. raimondii* (D_5 subgenome), most of which contained typical four conserved annexin repeats (Fig. S2). Then using *GhAnn2* as a BLAST query sequence in NCBI, we obtained a total of 250 ESTs from four cotton species (Table S1), including 156 ESTs from *G. hirsutum* ($(AD)_1$ genome), 15 ESTs from *G. barbadense* ($(AD)_2$ genome), 34 ESTs from *G. arboretum* (A_2 genome) and 45 ESTs from *G. raimondii* (D_5 genome). ESTs of each species were assembled respectively and 10 contigs were obtained, including two contigs of *Gh* (Ghcontig1, 2), 3 contigs of *Gb* (Gbcontig1, 2, 3), 2 contigs of *Ga* (Gacontig1, 2) and 3 contigs of *Gr* (Grcontig1, 2, 3) (Table S2 and S3). Then phylogenetic analysis was performed to examine the evolutionary relationship between the reported annexins and the annexins we found (14 *Gr* annexins and 10 annexin contigs). From the sequence analysis it became clear that *GhAnn2*, Ghcontig1, Gbcontig1, Grcontig1, Gacontig1 and Gorai.007G060900, two reported annexins *AnnGh2* and *AnnGh6* should be the orthologous of the same annexin ancestor in different cotton species (Fig. 1a; Table S4). According to this, contig2s, Gorai.007G239000 and the other three reported annexins (*GhAnx1*, *AnxGb1-2*) showed at least 99 % identity which

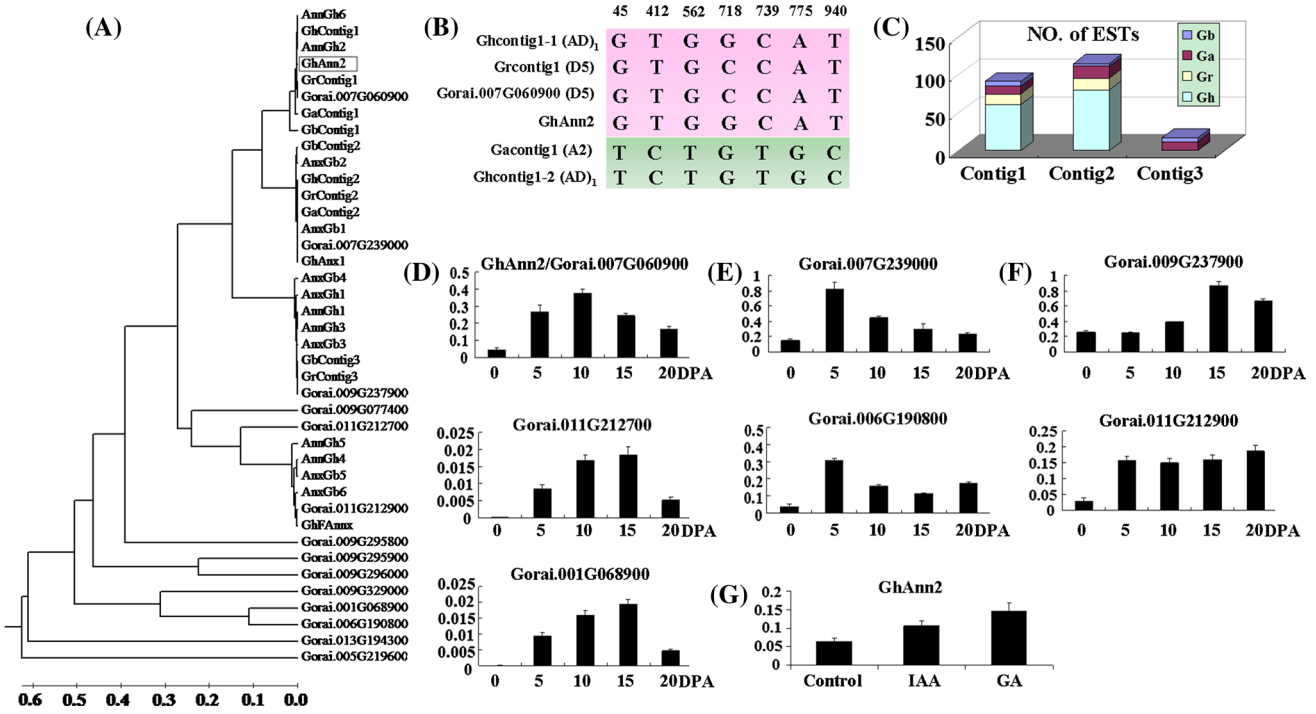

Fig. 1 Phylogenetic analysis and subgenomic location of *GhAnn2* and relative expression levels of cotton annexins in cotton fibers. **a** Phylogenetic analysis of annexins identified in this study and all the previously reported annexins. Phylogenetic analysis was performed with ClustalX program (version 1.83) (Thompson et al. 1997) and MEGA4 (Tamura et al. 2007) by the Neighbor-Joining method. GhAnx1, AY351650; AnxGh1, AAC33305; GhFAnnx, ACJ11719; AnnGh1, AAB67993; AnnGH2, AAB67994; AnnGh3-6, JX897059-62; AnxGb1-6, KC316004-9. **b** Subgenomic location of *GhAnn2*. ESTs of Ghcontig1 could be divided into two groups (Ghcontig1-1 and Ghcontig1-2) according to the SNP loci between Grcontig1 and Gacontig1. According to these SNP loci, *GhAnn2* was similar to Grcontig1 with only one nucleotide difference in the position 718, indicating that *GhAnn2* might come from D^T genome. The numbers such as 45, 412, 562 et al. represent the position of nucleotide in ORF of *GhAnn2* sequence. **c** The numbers of annexin ESTs from NCBI that assemble contigs 1, 2 and 3. **d–f** qRT-PCR analysis of the seven annexin family members expressed in fibers. According to the expression model in fiber, these members are divided into three groups. **d** The first group had highest levels in fiber cells at fast elongating phases (5–15 DPA). **e** The second group expressed to highest levels at 5 DPA. **f** The third group had highest expression levels in 20 DPA but also expressed to relatively high level in fiber cells at 5–15 DPA. Gorai.007G060900, Gorai.007G239000 and Gorai.009G237900 were the three members that had the highest expression levels in these three groups, respectively. *Gh UBQ7* (DQ116441) was included as the template control. The qRT-PCR results were obtained from three independent RNA extractions. *Error bars* are the SDs of four technical replicates. 0, ovules at 0 DPA; 5–20 DPA, fibers at 5–20 DPA. **g** qRT-PCR to show the expression of *AnnGh2* is induced by exogenous 5 μM IAA and 0.5 μM GA_3. Zero DPA ovules cultured in BT medium with 5 μM IAA and 0.5 μM GA_3 for 12 h were sampled for RNA extraction. Cotton *UBQ7* was included as the template control. The qRT-PCR results were obtained from three independent RNA extractions. *Error bars* are the SDs of four technical replicates

should also be orthologous in different cotton species (Fig. 1a, Table S5). Contig3, Gorai.009G237900 and the other four reported annexins (*AnxGh1*, *AnnGh1*, *AnnGh3* and *AnxGb3*) showed at least 98 % identity and formed another potential annexin family member in different cotton species (Fig. 1a, Table S6).

Sequence analysis showed *GhAnn2* had the best match with contig1 compared to contig2 and contig3. In order to survey the subgenomic location of *GhAnn2,* Grcontig1 and Gacontig1 were aligned first and some distinctive SNP loci were discovered (Fig. 1b). Then Ghcontig1 and its assembled ESTs were analyzed in detail and we found the ESTs could be divided into two groups (Ghcontig1-1 and Ghcontig1-2) according to the SNP loci between Grcontig1 and Gacontig1. Ghcontig1-1 matched Grcontig1 and Ghcontig1-2 matched Gacontig1 (Fig. 1b). The EST number ratio of the two groups was about 5:1. These results inferred that Ghcontig1 contained two genes from A and D sub-genome respectively and the one from Dt genome expressed in higher level than the one from At genome. According to SNP loci, *GhAnn2* was found to be similar to Grcontig1 with only one nucleotide difference in the position of 718 and might come from Dt genome (Fig. 1b). And *AnnGh2* and *AnnGh6* were also from Dt genome. Ghcontig1, Gbcontig1, Gacontig1 and Grcontig1 were assembled by a total of 91, contig2 by 151 ESTs and contig3 by only 20 ESTs from *Gb* and *Gr*, respectively (Fig. 1c; Table S3). Because these ESTs were mainly from fiber, the number of assembled

ESTs could reflect the relative expression level of the annexin gene represented by the contigs in fiber to some extent. According to this, the annexin genes represented by contig1, contig2 and contig3 would have relatively high expression level in cotton fiber. Therefore, besides *GhAnn2*, it is possible there are other annexin family members with relatively high expression level in cotton fiber.

In order to know the expression level of each of the annexin members, RT-PCR was firstly performed to analyze *Gr* annexin expression levels in various tissues of upland cotton plants. The results showed that about half of the annexin members were expressed in developing fiber cells (Fig. S3). These fiber-expressed annexins were further analyzed by qRT-PCR. GhAnn2/Gorai.007G060900, the other two annexin family members Gorai.007G239000 and Gorai.009G237900 were found to have the most abundant transcripts in fibers compared to the other cotton annexins. These three annexin genes showed highest similarities with contig1, contig2 and contig3 (Fig. 1a) and the expression levels measured by qRT-PCR of these three annexins in fibers were consistent with the ESTs redundancies (number of ESTs) of contig1, contig2 and contig3 (Fig. 1c). We divided all the *Gr* annexin members into three groups dependent on their expression model in fibers. The first group as shown in Fig. 1d contained three annexin members (Gorai.007G060900, Gorai.011G212700 and Gorai.001G068900) had highest level in fiber cells at fast elongating phase (5–15 DPA; Fig. 1d). Two members (Gorai.007G239000 and Gorai.006G190800) with highest levels at 5 DPA formed the second group (Fig. 1e). The last group contained Gorai.009G237900 and Gorai.001G212900 which had highest expression level at 20 DPA but also expressed to relatively high level in fiber cells at 5–15 DPA (Fig. 1f). Due to the relatively high expression levels in fibers at the fast elongating stage, GhAnn2/Gorai.007G060900 would be a good candidate for studying the role of annexin in fiber elongation.

Plant phytohormones such as IAA, GA_3 play essential roles in fiber development (Beasley and Ting 1973; Xiao et al. 2010; Zhang et al. 2011). To study the potential effects of these hormones on the expression of cotton annexin genes, we measured the expression level of *GhAnn2* in 0 DPA ovules cultured for 12 min in BT medium (Beasley and Ting 1973) with or without 5 μM IAA or 0.5 μM GA_3. qRT-PCR showed that when treated with IAA or GA_3 the relative expression level of *GhAnn2* was nearly double and triple that of the control, respectively (Fig. 1g).

RNAi silencing of *GhAnn2* results in shorter and thinner fibers

GhAnn2 was highly expressed in elongating fibers and could be induced by IAA and GA_3, which implied that it may play a role in fiber development. To examine the exact effect of *GhAnn2* on fiber development, an RNAi silencing construct against *GhAnn2* was transformed into upland cotton variety YZ1. Three independent transgenic *GhAnn2* RNAi cotton lines were obtained for further analysis. After analysis by qRT-PCR and Northern blots, two RNAi lines (i21 and i34) were found to have much lower expression level of *GhAnn2,* compared with wild type (Fig. 2a, b). Southern blotting confirmed that they are the positive transforms (T2) (Fig. S4). Because there were other annexin members expressed to high levels in fiber, RT-PCR was also carried out to check whether those, other than *GhAnn2*, were affected in their expression in the RNAi lines. The analyses revealed no obvious changes in the expression of other annexins (Fig. S5).

Fiber length, a key determinant of fiber quality and output, was compared between the RNAi lines and wild-type. As shown in Fig. 2c, d, the development of *GhAnn2* RNAi fiber cells was significantly inhibited. The length of 5 DPA fiber cells of i21 and i34 lines were 2.9 ± 0.3 and 3.1 ± 0.2 mm, decreased by 14.7 and 10.1 %, respectively, in comparison with that of wild type. At 10 DPA, fiber cells of i21 and i34 lines only elongated to 13.0 ± 1.1 and 12.6 ± 0.8 mm, 12.9 and 15.6 % shorter than the wild-type, respectively. The length of fiber cells at 15 DPA (21.0 ± 1.0 and 21.6 ± 1.0 mm) was also significantly shorter for i21 and i34 lines, respectively, than the wild-type (23.3 ± 0.8 mm), showing a decrease of 9.8 and 7.1 %, respectively. This reduced fiber length phenotype was also observed at maturity. The mature fiber final length of i21 and i34 lines were only 26.4 ± 0.5 and 26.2 ± 0.7 mm, which is 8.7 and 9.4 % shorter than the wild-type (29.0 ± 0.6 mm). qRT-PCR was performed to measure the relative expression levels of several fiber elongation related genes such as *GhEXPA1* (GenBank: ABD48785; Xu et al. 2013); *GhPEL* (GenBank: DQ073046.1; Wang et al. 2010a, b), *GhPME1* (GenBank: JQ340871.1; Liu et al. 2013). These genes' expression levels were decreased both in 5 DPA and 10 DPA fibers of *GhAnn2* RNAi lines compared to the wild-type, especially the *GhEXPA1* gene (Fig. 2e). The results above indicate that *GhAnn2* expression is required for normal fiber elongation. It is worth to mention that the RNAi lines showed normal vegetative phenotypes (Fig. S6), suggesting that the silencing effect appears to be fiber specific.

Micronaire is an indicator of fiber maturity and fineness, which is one of the most important fiber characteristics for international cotton classers and spinners. It is a measure of the rate at which air flows under pressure through a plug of lint of known weight compressed into a chamber of fixed volume. The rate of air flow depends on the resistance offered by the total surface area of the fibers which is related to the linear density as well as the thickness of the fiber walls. A reduction in linear density, wall

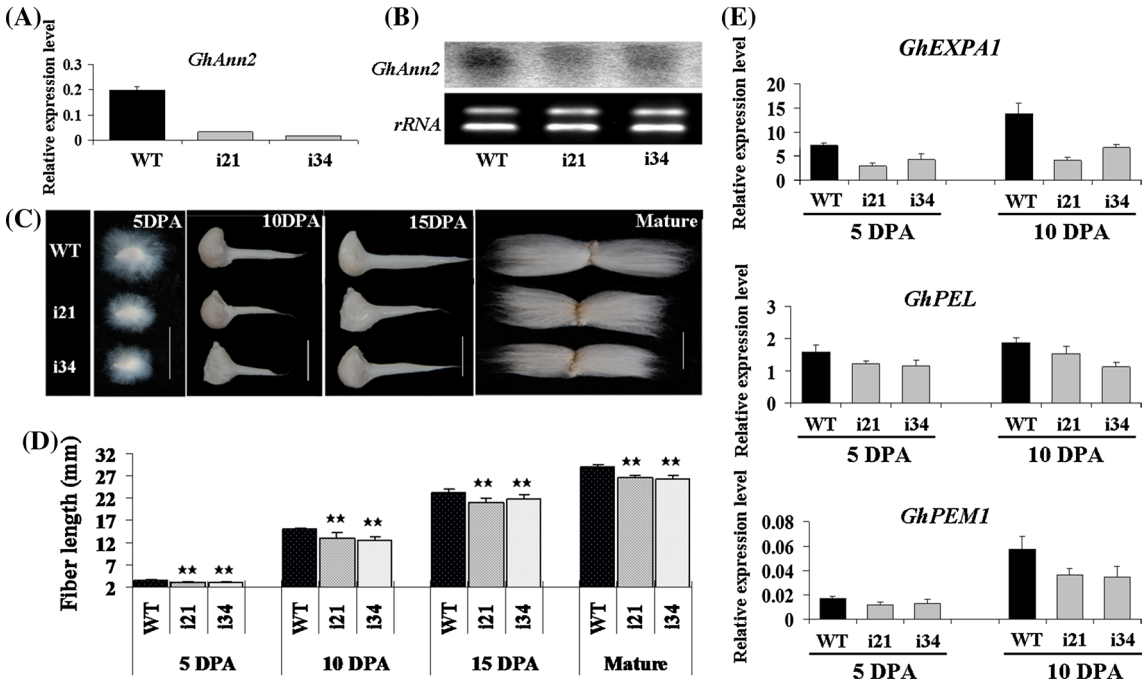

Fig. 2 Down-regulating *GhAnn2* inhibits fiber elongation. **a** qRT-PCR analysis of *GhAnn2* in 10 DPA fibers of *GhAnn2* RNAi lines and wild-type. The expression level of *UBQ7* was used as the internal control to standardize the RNA samples for each reaction. *Error bars* are the SDs of four technical replicates. **b** Northern blotting analysis of *GhAnn2* with 10 DPA fibers. rRNA was used as an internal standard. **c** Straight fibers of 5 DPA, 10 DPA, 20 DPA and mature fibers from two RNAi lines and wild type. 5 DPA, *bar* = 1 mm; 10 DPA, 15 DPA and mature, *bar* = 10 mm. **d** Fiber length measurements for the whole periods of fiber development. The *bar* represents the SD of at least 40 ovules in 4 bolls. $^{**}P < 0.01$; $^{*}P < 0.05$. **e** qRT-PCR analysis of fiber elongation related genes in 5 and 10 DPA fibers from two RNAi lines and wild type. The expression level of *UBQ7* was used as the internal control to standardize the RNA samples for each reaction. *Error bars* are the SDs of four technical replicates. WT, wild type; i21, i34, two independent RNAi lines

thickness or fiber perimeter decreases the micronaire reading (Montalvo and Hoven 2005). Mature fibers of *GhAnn2* RNAi lines and wild type were collected at the same time and the same position of the plants and used for micronaire measurement. The result showed that the fiber micronaire values were considerably lower in *GhAnn2* RNAi transgenic lines (5.01 ± 0.31 and 4.97 ± 0.28 for i21 and i34, respectively) than the wild-type (5.64 ± 0.24) (Fig. 3a). In order to confirm the result above, paraffin histological sections were made of mature fibers from wild-type and transgenic lines and observed under the light microscope. The cell walls of mature fibers in RNAi lines appeared to be thinner than those of wild-type. Measurements of the fiber wall thickness showed that fiber cell wall of *GhAnn2* RNAi lines were 3.31 ± 0.25 and 2.76 ± 0.27 μm, significantly thinner than that of wild type (3.85 ± 0.33 μm) (Fig. 3b). To investigate the molecular basis of this, qRT-PCR was performed to analyze the expression levels of genes associated with the deposition of the secondary cell wall cellulose including *GhCelA1* (cellulose synthase; Shimizu et al. 1997; Li et al. 2013a) and *GhCTL1* (chitinase-like proteins; Zhang et al. 2004). These genes were found to be down-regulated both in 15 and 20 DPA fibers of *GhAnn2* RNAi lines (Fig. 3c, d), which is consistent with the reduced cell wall thickness phenotypes.

RNAi silencing of *GhAnn2* decreases Ca^{2+} influx at the apex of fiber cells

In vitro studies of certain plant annexins have revealed their potential to act directly as calcium channels (Mortimer et al. 2008). In order to test whether *GhAnn2* is this kind of annexin, the non-invasive, scanning ion-selective electrode technique was used to measure the Ca^{2+} influx at the fiber cell apex from the extracellular into intracellular environment. The result showed that at 5 DPA, there was a Ca^{2+} influx at the tips of wild type fiber cells (Fig. 4a). The rate was 31.1 ± 6.4 pmol. cm^{-2}. s^{-1}. However, there was no Ca^{2+} influx at the tip of *GhAnn2* RNAi fibers, instead, there was Ca^{2+} efflux from the intracellular into extracellular environment (Fig. 4a). The efflux rates were 29.2 ± 4.4 and 58.6 ± 6.4 pmol. cm^{-2}. s^{-1} for i21 and i34 lines, respectively (Fig. 4b). A previous study has shown that Ca^{2+} influx is important for fiber elongation (Tang et al. 2014). In order to confirm the essential role of tip Ca^{2+} influx in cotton fiber cell elongation, an ovule culture assay was

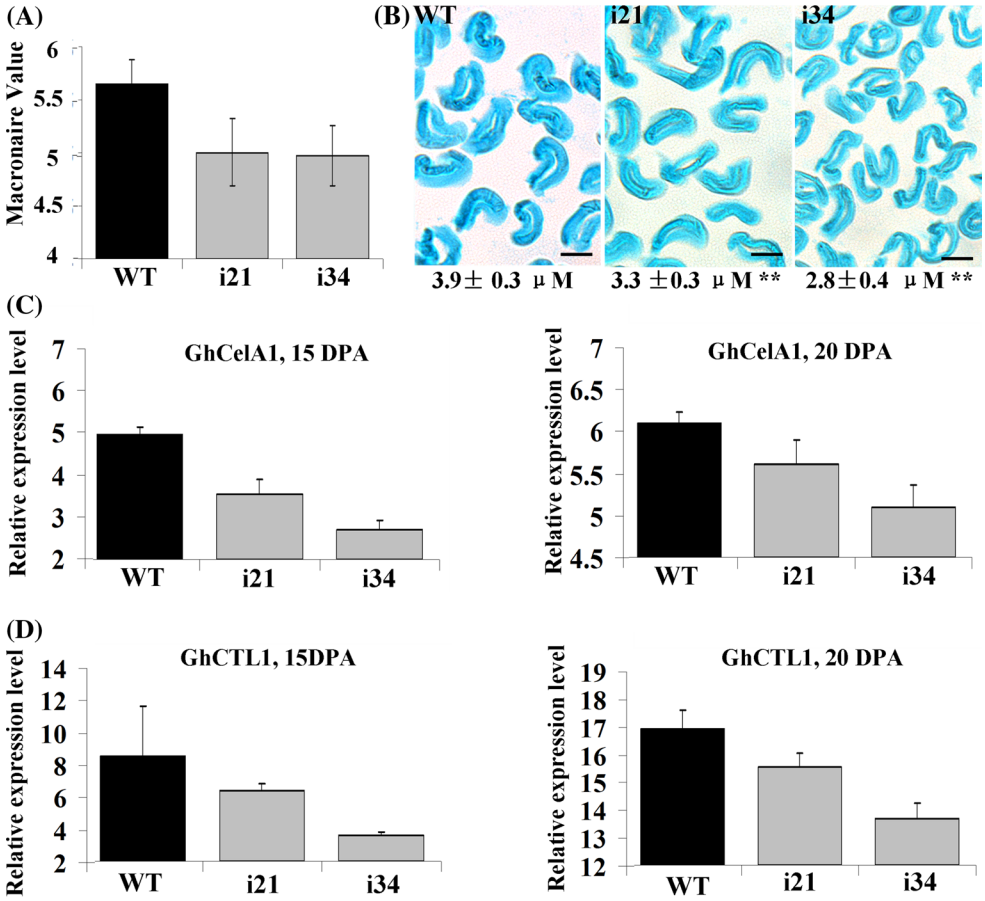

Fig. 3 Down-regulating *GhAnn2* inhibits fiber secondary cell wall synthesis. **a** Micronaire values of mature fiber in *GhAnn2* RNAi lines and wild type. *Error bars* indicate the SD of three biological replicates. **b** Paraffin sections of mature fiber cell wall. The data below show the thickness of the fiber cell wall (μm). *Bar* = 10 μm. $^{**}P < 0.01$. **c** qRT-PCR analysis of *GhCelA*1 in 15 and 20 DPA fibers from *GhAnn2* RNAi lines and wild type. The expression level of *UBQ7* was used as the internal control to standardize the RNA samples for each reaction. *Error bars* are the standard deviations of four technical replicates. **d** qRT-PCR analysis of *GhCTL1* in 15 and 20 DPA fibers from *GhAnn2* RNAi lines and wild type. The expression level of *UBQ7* was used as the internal control to standardize the RNA samples for each reaction. *Error bars* are the SDs of four technical replicates

performed and the results showed that when 7 and 28 μM Ca^{2+} channel blocker La^{3+} (Yuasa et al. 1998) was applied, fiber elongation was significantly inhibited (Fig. S7). Then we examined the effect of La^{3+} on RNAi lines fibers. When ovules were cultured in BT medium (containing 5 μM IAA and 0.5 μM GA_3) for 10 days, the wild type fiber cells could elongate to 10.5 ± 0.3 mm, whereas the fiber of RNAi lines could only elongate to 8.3 ± 0.5 mm, 7.9 ± 0.3 mm for i21 and i34, respectively, consistent with the length data of mature fiber in vivo. Upon addition of 14 μM La^{3+} to the medium, the lengths of both wild-type and RNAi fibers were decreased. However, the fiber lengths of i21 and i34 lines decreased 63.9 and 68.4 %, respectively, which were significantly shorter than that of wild-type (decreased 34.3 %). The results suggest that *GhAnn2* fibers of RNAi lines were more sensitive to La^{3+} (Fig. 4c).

RNAi silencing of *GhAnn2* down-regulates expression of Ca^{2+} sensors in elongating fiber cells

Ca^{2+} signaling plays crucial roles in a wide array of growth and developmental processes (Jones and Lunt 1967). Ca^{2+} influx can contribute to the generation of Ca^{2+} signals which can be sensed by Ca^{2+} sensors, including CaMs (calmodulins)/CMLs (CaM-like proteins), CDPKs (Ca^{2+}-dependent protein kinases)/CCaMK (calcium/calmodulin-dependent protein kinases), and CBLs (calcineurin B-like proteins; Kudla et al. 2010). To test if silencing *GhAnn2* may affect Ca^{2+} signaling, we searched the D genome Phytozome 9.1 database for genes encoding Ca^{2+} sensors. The exercise identified 18 Ca^{2+} sensor genes that had relatively high expression level in elongating fibers (data not shown). We examined the expression levels of these genes in 15 DPA fibers of *GhAnn2* RNAi lines and wild type. The results showed that the expression levels of 2 *CaMs*, 4 *CMLs*, 4 *CCaMKs* and 2 *CBLs* were down-regulated in *GhAnn2* RNAi fibers compared to the wild type, especially regarding to the two CaMs and CBLs. The data provided further evidence that GhAnn2 may have an effect on Ca^{2+} influx and signaling during fiber development (Fig. 5).

Discussion

Annexins typically form a big gene family in all multicellular organisms. Genome sequencing revealed that there are eight annexin genes in *Arabidopsis* (Cantero et al. 2006; Mortimer et al. 2008), nine in rice (Moss and Morgan

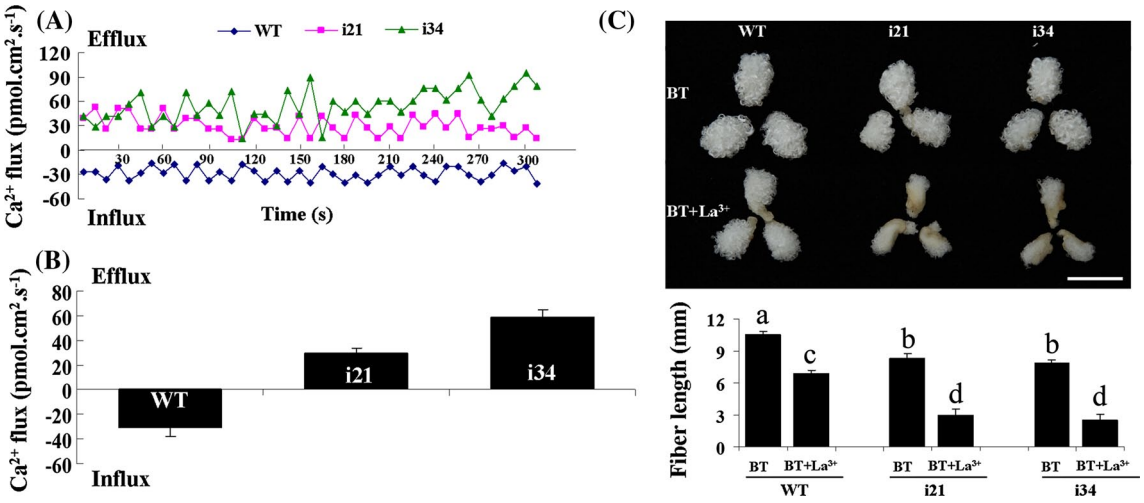

Fig. 4 RNAi silencing of *GhAnn2* decreases the rate of Ca^{2+} influx at the fiber cell apex. **a** Tip Ca^{2+} flux of 5 DPA fiber cells was measured by noninvasive scanning ion-selective electrode. Four bolls in one line were analyzed, and at least three fiber cells in one boll were tested. **b** Average rate of Ca^{2+} flux of 5 DPA fiber cells. *Error bars* indicate the SD of three biological replicates. **c** La^{3+} treatments in ovule cultures of *GhAnn2* RNAi and wild type. 0 DPA ovules of *GhAnn2* RNAi lines and the wild type were cultured in BT medium and BT medium containing 28 μM La^{3+} for 10 days. More than 10 cultured ovules per treatment were used for fiber length measurement. Different letters (*a*, *b*, *c*, *d*) in the histograms indicate statistically significant differences at $p < 0.05$ based on ANOVA (Student–Newman–Keuls method)

2004) and ten in Medicago (Talukdar et al. 2009). In cotton, a draft genome of *G. raimondii* (D_5 subgenome) has been sequenced and assembled recently (Wang et al. 2012). We have integrated the genome and identified 14 annexin genes in *G. raimondii* (D_5 genome; Fig. 1a, S2). We found that many reported annexin genes with different names were in fact the same genes in different cotton species or cultivars (Fig. 1a; Table S4-6) and some small differences in nucleotide sequence might have come from SNP between the A and D genome or sequencing errors. The observation that annexin proteins constitute up to 0.1 % of total plant proteins (Delmer and Potikha 1997; Clark et al. 2001; Moss and Morgan 2004; Mortimer et al. 2008) indicates their essential role in plant cell development. However, definitive evidence of the *in planta* role of annexin are still lacking thus far.

Developing cotton fiber is a fast-growing single cell which may be enriched for annexins. This was observed in our RT-PCR and qRT-PCR studies. About half of the annexin family members were expressed in cotton fiber and three members including Gorai.007G239000, Gorai.007G060900 and Gorai.009G237900 had relatively high expression levels compared to other members (Fig. 1d–f). Although several studies have been conducted to examine their expression levels in cotton and potential roles in *Arabidopsis* (Shin and Brown 1999; Tu et al. 2007; Wang et al. 2010a, b; Zhou et al. 2011; Li et al. 2013a; Huang et al. 2013), the mechanism by which annexins regulate cotton fiber development is still unknown.

In this study, by using transgenic and cell biology approaches, we provide evidence that *GhAnn2* may contribute to Ca^{2+} influx at the tip of fiber cells (Fig. 4; Fig. S7). Ca^{2+} plays crucial roles in a wide array of growth and developmental processes (Jones and 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 achieved by Ca^{2+} influx from the extracellular environment maintains the rate of cell elongation in pollen tubes (Hepler et al. 2001; Holdaway-Clarke and Hepler 2003). Ca^{2+} has been reported to play important roles in fiber elongation (Lee et al. 2007; Taliercio and Boykin 2007; Padmalatha et al. 2012; Walford et al. 2012; Tang et al. 2014). A high Ca^{2+} gradient is found in the cytoplasm of fast elongating cotton fiber cells near the growing tip (Huang et al. 2008). During fiber development, there is a sustained Ca^{2+} influx at the tip of fiber cells and the flux rate peaks during fiber rapid elongating at between 10 and 15 DPA (Tang et al. 2014) when plasmodesmata close and all solutes including ions such as Ca^{2+} must move into fibers across plasma membrane (Ruan 2007). We found inhibition of Ca^{2+} influx by Ca^{2+} channel blocker La^{3+} (Yuasa et al. 1998) and Ca^{2+} pool release channel blocker 2-APB (Bootman et al. 2002) strongly suppresses fiber cell elongation (Fig. 4c, S6; Tang et al. 2014), supporting an essential role for Ca^{2+} in fiber development. On the other hand, Ca^{2+} influx contributes to the generation of Ca^{2+} signal which should be sensed by Ca^{2+} sensors, such as CaMs/CMLs, CDPKs/CCaMKs,

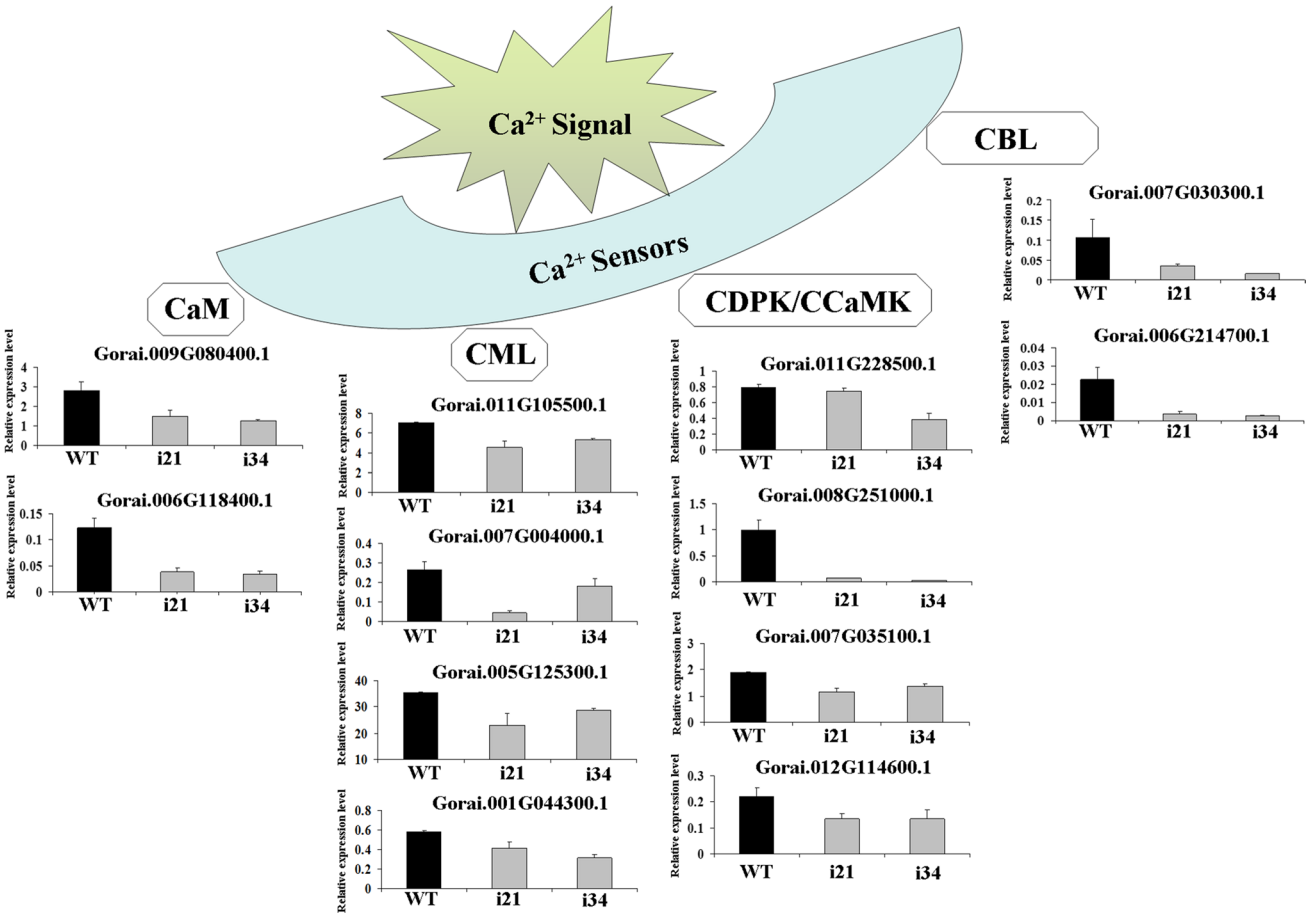

Fig. 5 RNAi silencing of *GhAnn2* down-regulated expression levels of Ca^{2+} sensors in elongating fiber cells. CaMs (calmodulins)/CMLs (CaM-like proteins), CDPKs (Ca^{2+}-dependent protein kinases)/CCaMK (calcium/calmodulin-dependent protein kinases), and CBLs (calcineurin B-like proteins) are the three largest categories of Ca^{2+} sensors in plants. The numbers begin with Gorai are the Ca^{2+} sensor genes in *G. raimondii*. The expression level of *UBQ7* was used as the internal control to standardize the RNA samples for each reaction. *Error bars* are the SDs of four technical replicates

and CBLs (Kudla et al. 2010). There are several reports that genes associated with calmodulin and calmodulin-binding proteins are up-regulated in fiber initials and during fiber elongation by expression profile analysis (Lee et al. 2007; Taliercio and Boykin 2007). Consistent with these reports, two calmodulins including *GhCaM7* (Gorai.009G080400.1) and Gorai.006G118400.1 were down-regulated in the *GhAnn2* RNAi fibers. *GhCaM7* had the highest expression level in elongating fibers among all the *GhCaM* genes. Similar to *GhAnn2*, suppression of *GhCaM7* by RNAi also inhibits fiber elongation (Tang et al. 2014) and cell wall synthesis (Fig. S8). Except for calmodulin, other Ca^{2+} sensors that had relatively high expression levels in elongating fibers are also down-regulated in *GhAnn2* RNAi fibers. Although the function of these CDPKs/CCaMKs, and CBLs in cotton fiber development remains to be determined, studies in pollen tubes shows that these Ca^{2+} sensors play important roles in cell tip-growth (Zhao et al. 2013).

Annexins are small proteins that possess structural motifs that could support calcium transport. Several animal annexins have been reported to function in vitro as Ca^{2+} channels, such as the vertebrate annexins A1, 2, 5–7, and 12 (Burger et al. 1994; Liemann et al. 1996; Kourie and Wood 2000). Some loss-of-function mutants of annexin have impaired ability to regulate cytoplasmic Ca^{2+}. These include, for example, A5 (2/2) chicken DT40 cells (Kubista et al. 1999), A7 (+/2) murine brain cells (Watson et al. 2004), and A7 (2/2) murine cardiomyocytes (Schrickel et al. 2007). In plants, a *Zea mays* annexin could increase $[Ca^{2+}]_{cyt}$ of *Arabidopsis* protoplasts when the recombinant annexin was added to the root protoplasts expressing the calcium sensor protein, aequorin (Demidchik et al. 2002; Dodd et al. 2006). The pharmacological profile was consistent with annexin activation (at the extracellular plasma membrane face) of *Arabidopsis* Ca^{2+}-permeable nonselective cation channels. It is suggested that annexins create a Ca^{2+} influx pathway directly, particularly during stress

responses involving acidosis (Laohavisit et al. 2009). In addition, the *Arabidopsis* loss-of-function mutant (Atann1) that lacks root hairs was found to be impaired in OH^- activated Ca^{2+}- and K^+-permeable conductance. And the OH^--activated Ca^{2+} conductance could be reconstituted by recombinant ANN1 in planar lipid bilayers. ANN1 therefore presents as a novel Ca^{2+}-permeable transporter in plants (Laohavisit et al. 2012). In cotton, we found that GhAnn2 mediates the tip Ca^{2+} influx (Fig. 4a, b). Together with previous studies, it is suggested that *GhAnn2* might also act as a Ca^{2+} channel and have a positive role in fiber elongation.

In summary, by taking a transgenic approach, we show that GhAnn2 is required for fiber cell elongation through mediating Ca^{2+} influx at the tip of fiber cells and possibly contributes to the generation of Ca^{2+} signal.

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