RESEARCH PAPER

GhCFE1A, a dynamic linker between the ER network and actin cytoskeleton, plays an important role in cotton fibre cell initiation and elongation

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Abstract

Fibre cell initiation and elongation is critical for cotton fibre development. However, little is known about the regulation of initiation and elongation during fibre cell development. Here, the regulatory role of a novel protein GhCFE1A was uncovered. *GhCFE1A* is preferentially expressed at initiation and rapid elongation stages during fibre development; in addition, much higher expression of *GhCFE1A* was detected at the fibre initiation stage in fibreless cotton mutants than in the fibre-bearing TM-1 wild-type. Importantly, overexpression of *GhCFE1A* in cotton not only delayed fibre cell elongation but also significantly reduced the density of lint and fuzz fibre initials and stem trichomes. Yeast two-hybrid assay showed that GhCFE1A interacted with several actin proteins, and the interaction was further confirmed by co-sedimentation assay. Interestingly, a subcellular localization assay showed that GhCFE1A resided on the cortical endoplasmic reticulum (ER) network and co-localized with actin cables. Moreover, the density of F-actin filaments was shown to be reduced in *GhCFE1A*-overexpressing fibres at the rapid elongation stage compared with the wild-type control. Taken together, the results demonstrate that GhCFE1A probably functions as a dynamic linker between the actin cytoskeleton and the ER network, and plays an important role in fibre cell initiation and elongation during cotton fibre development.

Key words: Actin cytoskeleton, cotton fibre, endoplasmic reticulum, fibre elongation, fibre initiation, GhCFE1A.

Introduction

Cotton is the primary renewable source of natural textile fibre. Cotton fibres are single-celled trichomes derived from the ovule epidermis which are morphologically divided into two types: lint and fuzz. About 25% of the epidermal cells of the cotton seed coat differentiate and develop into unicellular long and mature fibres (lint) that can be spun into yarn (Basra and Malik, 1984; Kim and Triplett, 2001). In *Gossypium hirsutum*, lint fibre cells extend from the seed coat either before or on the day of anthesis, with a final length of ~30 mm, whereas fuzz fibres initiate a few days later and fail

to elongate appreciably (<5 mm; Kim and Triplett, 2001; Lee *et al.*, 2006, 2007).

Cotton fibre development undergoes four distinct but overlapping stages: fibre initiation, elongation, secondary cell wall biosynthesis, and drying and maturation (Basra and Malik, 1984; Ruan and Chourey, 1998). The number of fibre cells per ovule is established in the initiation phase, and the length and strength of fibre are determined mainly during the stages of elongation and secondary cell wall synthesis. During cotton fibre elongation, the thin actin arrays

and thick and long actin cables and bundles parallel to the growing axis of the fibre cells form a complicated network, which orchestrates the trafficking of secretory vesicles and other large organelles and plays a critical role in the process of fibre elongation (Li *et al.*, 2005; J. Wang *et al.*, 2010). A previous study showed that 15 *GhACT* cDNAs encoding putative actin proteins were found to be differentially expressed in various tissues. Specifically, *GhACT1* is predominantly expressed in fibre cells, and its suppression by RNA interference (RNAi) disrupted the actin cytoskeleton network, causing reduced fibre elongation (Li *et al.*, 2005). In addition, the dynamic actin cytoskeleton is regulated by a number of actin-binding proteins. Cotton LIM domain-containing proteins were reported to play important roles in modulating the actin cytoskeleton (Han *et al.*, 2013; Li *et al.*, 2013, 2014). GhPLIM1 and GhWLIM5 can directly bind actin and bundles F-actin *in vitro* (Li *et al.*, 2013, 2014); indeed, WLIM1a plays dual roles during cotton fibre development, acting as a actin bundler to promote cell elongation at the fibre cell elongation stage and as a transcriptional factor to trigger lignin biosynthesis at the secondary cell wall biosynthesis stage (Han *et al.*, 2013). AnxGb6 was reported to interact with actin GbACT1 (Huang *et al.*, 2013). In addition, overexpression of an actin-binding protein-encoding gene, *GhPFN2*, caused early terminatation of cell elongation, producing obvious short fibres (J. Wang *et al.*, 2010), and down-regulation of the actin depolymerizing factor-encoding gene *GhADF1* resulted in a greater abundance of actin filaments in the cortical region of the transgenic fibre cells, producing fibres with increased length and strength (Wang *et al.*, 2009).

It is well known that the actin cytoskeleton plays a crucial role in endoplasmic reticulum (ER) movement and distribution in plant cells (Voeltz *et al.*, 2002; Borgese *et al.*, 2006; Sparkes *et al.*, 2009). The cortical ER, which is a highly dynamic structure in plants composed of a polygonal tubular network, was first described by electron microscopists in the 1960s (Porter and Machado, 1960; Sparkes *et al.*, 2009, 2011). In *Arabidopsis thaliana*, mutations of the membrane-bound GTPase ROOT HAIR DEFECTIVE3 (RHD3), which was reported to mediate ER fusion maintaining ER morphology, caused defects in cell expansion and elongation, resulting in a short root and short, wavy root hairs (Wang *et al.*, 1997, 2002; Stefano *et al.*, 2012; Zhang *et al.*, 2013); another study reported that maMYB, a member of the plant-specific R2R3-MYB family, localized to the ER, and silencing of *maMyb* led to a reduction of root hair length (Slabaugh *et al.*, 2011). However, thus far, there is little known about the role of the ER network in cotton fibre cell development.

In this study, a gene with unknown function was isolated and designated as *GhCFE1A*, which is preferentially expressed at the initiation and elongation stage during cotton fibre development. Interestingly, it was found that GhCFE1A decorates the ER network and also binds to actin cables. Furthermore, overexpression of *GhCFE1A* resulted in reduced density of lint and fuzz fibre initials and impaired fibre elongation.

Materials and methods

Plant materials

Fibre-bearing wild-type (7235, TM-1) *G. hirsutum* and three fuzzless–lintless cotton mutants (MD17, SL1-7-1, and Xu142 fl), *G. barbadense* (Hai7124), *G. herbaceum*, and *G. raimondii* were field-cultivated in Nanjing, China, using normal cotton farming practices. Developing ovules and fibres were excised from flower buds or bolls and ovules on selected days before or after anthesis relative to the day of anthesis (0 DPA). Roots, stems, and leaves were collected from 2-week-old seedlings cultured in a growth chamber. All frozen materials were stored at –70 °C.

Gene cloning and sequence analysis

The full-length *GhCFE1A* cDNA sequence was isolated from a 7235 cDNA library, which was constructed as described previously (H.H. Wang *et al.*, 2010). To obtain the genomic sequences of *GhCFE1* homeologues in TM-1, Hai7124, and two diploid progenitor cotton species, *G. herbaceum* L. (A genome), and *G. raimondii* Ulbrich (D genome), primers (*GhCFE1*-full-F/R) were designed to amplify the open reading frame (ORF) using the genomic DNA as a template. All the fragments were sequenced after they were cloned into T-vectors. The conserved protein domains were searched in the National Center for Biotechnology Information (NCBI) database. The GhCFE1A signal peptide was predicted using the web-based program SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). For sequence alignment analysis, the genomic and cDNA sequences and peptides of GhCFE1 homeologues were aligned with ClustalX (Thompson *et al.*, 1997) and, for phylogenetic analysis, a phylogenetic tree was constructed with MEGA5 software (Tamura *et al.*, 2011).

Southern blot hybridization and molecular mapping

Genomic DNA isolation and Southern blotting were carried out according to the methods described by H.H. Wang *et al.* (2010). The probe fragment for detecting the copy number was amplified by *GhCFE1* primers (*GhCFE1*-RT-F/R) containing one *Xba*I restriction site, and genomic DNA of TM-1 was digested with *Hin*dIII, *Eco*RI, and *Xba*I restriction enzymes. Transgenic cotton plants were detected using the *NPTII* fragment as a probe, and genomic DNA was digested with *Eco*RI.

GhCFE1D was mapped using the BC_1 [(TM-1×Hai7124)×TM-1] interspecific mapping population. The pair of primers consisting of amplified regions with Dt subgenome polymorphisms between TM-1 and Hai7124 was used to survey 138 individuals of the BC_1 mapping population; however, there were no At subgenome polymorphisms between the two mapping parents. The polymorphic loci were integrated in the backbone map (Guo *et al.*, 2008) by JoinMap version 3.0 (Van Ooijen and Voorrips, 2001).

RT–PCR and qRT-PCR

The expression levels of *GhCFE1* homeologues were analysed by reverse transcription–PCR (RT–PCR) or real-time quantitative RT–PCR (qRT-PCR). The cotton elongation factor gene *GhEF1α* was used as a standard control in the RT–PCR. In the qRT-PCR, expression was normalized against the expression of *His3*. Total RNA was extracted using the cetyl trimethylammonium bromide (CTAB)–acidic phenol extraction method (Jiang and Zhang, 2003). A total of 2 μg of RNA per reaction from different tissues was reverse-transcribed from an oligo(dT)$_{18}$ primer using M-MLV reverse transcriptase (Promega, Madison, WI, USA; Cat# M1701) according to the manufacturer's recommendations. The cDNA samples were diluted 10-fold and 2 μl of the dilution was used as the template. qRT-PCR was performed with 40 cycles using a LightCycler FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland) in an

ABI7500 sequence detection system according to the manufacturer's protocol (Applied Biosystems, http://www.appliedbiosystems.com). For all reactions, three technical replicates were performed in each of the three biological experiments. The gene-specific primers used in the study are listed in Supplementary Table S1 available at *JXB* online. The relative expression level of the *GhCFE1* homeologues was calculated by the equation $Y=2^\wedge-\Delta Ct$ (where ΔCt is the difference between the Ct values of the *His3* products and the *GhCFE1* homeologue product; i.e. $\Delta Ct=Ct_{GhCFE1}-Ct_{His3}$.). The relative expression levels of transcription regulators were determined by the $\Delta\Delta Ct$ method (Livak and Schmittgen, 2001).

Plasmid construction and plant transformation

Sense and antisense *GhCFE1A* plant expression vectors were constructed using two pairs of primers with *Bam*HI (*Sac*I) and *Sac*I (*Bam*HI) (enzymes denoted in parentheses were used to construct the antisense vectors and the enzymes outside the parentheses were used for constructing the sense vectors) restriction sites. The amplified sense *GhCFE1A* product was inserted into the pBI121 and pBI121-E6 [a pBI121 derivative where the *Cauliflower mosaic virus* (CaMV) 35S promoter was replaced by a *GhE6* promoter] vectors at the *Bam*HI/*Sac*I sites to replace the *GUS* (β-glucuronidase) gene, while the amplified antisense *GhCFE1A* product was only inserted into the pBI121-E6 vector. The constructed vectors and pBI121 were transformed into *G. hirsutum* accession W0 by *Agrobacterium tumefaciens* strain LBA4404 (Li *et al.*, 2009). The transformed plants containing the empty vector pBI121 were used as the null control.

Heterologous expression of GhCFE1A in Escherichia coli *and co-sedimentation assay*

After removing the predicted signal peptide (amino acids 1–26), the remaining GhCFE1A sequence was inserted into the *Bam*HI/*Xho*I sites of the pET-30a (+) vector (Qiagen, Valencia, CA, USA), resulting in translational fusion of the protein with six histidine residues at the N-terminus. The *E. coli* strains carrying pET-30a (+)-*GhCFE1A* were cultured until the optical density at 600 nm (OD_{600}) reached 0.4. Then isopropyl-β-ᴅ-thiogalactopyranoside (IPTG, 0.6 mM) was added to induce the expression of the fusion protein. The proteins obtained in the induced and uninduced *E. coli* cultures were analysed using SDS–PAGE. His-tagged GhCFE1A proteins were purified using nickel–nitrilotriacetic acid resin following the procedures described by the manufacturer (Qiagen). As the purified proteins consisted of two bands with different size proteins in SDS–PAGE, GhCFE1A was digested with *Bam*HI and *Xho*I and cloned into the pGEX-6P-1 vector (GE Healthcare). Glutathione *S*-transferase (GST)–CFE1A recombinant proteins were expressed in *E. coli* following similar procedures to those described above and purified using glutathione–Sepharose following the procedures described by the manufacturer (GE Healthcare). A co-sedimentation assay was conducted according to Han *et al.* (2013). The proteins in the supernatants and pellets were separated by SDS–PAGE.

Protein extraction, quantification, and immunoblot analysis

Total proteins were extracted from the ovules, fibres, and leaves using plant protein extraction buffer [40 mM HEPES pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 0.4 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF)] and quantified by the Bradford method (Bradford, 1976). Equal amounts of protein for transgenic and wild-type plants were subjected to SDS–PAGE followed by western blot analysis. A polyclonal antiserum was raised in rabbits against a synthetic peptide corresponding to residues 233–246 (HLKKSDTWENHGRD) of GhCFE1A (GenScript). Corresponding secondary IRDye 800CW-labelled goat anti-rabbit IgG (H+L) antibodies were used for detection.

Scanning electron microcopy and fibre quality measurement

To compare the lint and fuzz fibre initiation difference between transgenic and wild-type plants, ovules and seeds were collected at 0 and 4 DPA, respectively, from similar positions on the cotton plants and fixed in 3% (v/v) glutaraldehyde (pH 7.2). After serial dehydration for 30 min each in 30, 50, 70, 80, 90, and 100% ethanol solutions, the samples were transferred into isoamyl acetate and dried at the critical point. The dried cotton ovules at 0 DPA and seeds at 4 DPA with the lint fibre removed were sputter-coated with gold on the surface, and then viewed and photographed with a scanning electron microscope (SEM; Hitachi S-3000N). After cotton ginning and cottonseed delinting, three independent fibre samples and cotton seeds (with or without fuzz) from each T_3 generation of the transgenic lines and wild-type cotton were weighed and counted to determine the lint index (fibre weight of 100 seeds) and the fuzz index (fuzz weight of 100 seeds). The fibres were then sent to the Center of Cotton Fibre Quality Inspection and Testing, Ministry of Agriculture (Anyang, Henan Province, China) to determine the quality of the cotton fibre. Seeds at 8, 10, 13, 15, 18, 20, 23, and 25 DPA were chosen to measure the fibre length for the dynamic curve analysis of fibre development, as reported previously (Gipson and Ray, 1969). Data were processed in Microsoft Excel using Student's *t*-test.

Yeast two-hybrid screening

The coding sequences of *GhCFE1A* with the signal peptide removed and partial sequence-containing domain conserved were amplified using gene-specific primers (Supplementary Table S1 at *JXB* online), and fused with the GAL4-DNA-binding domain in the pGBKT7 vector (Clontech) for constructing the bait vectors. A prey library of cotton ovules and fibres (0 DPA ovules; 5, 10, 15, and 20 DPA fibres) was constructed by fusing cDNAs with the GAL4-activation domain in the pGADT7-Rec vector (Clonetech). The yeast two-hybrid assay was performed according to the manufacturer's instructions. The plasmids of positive clones were extracted, and the cDNA sequences integrated in these plasmids were sequenced. The full length of the interacting genes was fused with the GAL4-activation domain in the pGADT7 vector (Clontech), and co-transformed into *Saccharomyces cerevisiae* AH109 with the bait vector to test their interaction.

Co-localization assay and live cell imaging analysis

The ORF of *GhCFE1A* was amplified and cloned into pGWB5 and pGWB6 to obtain the CFE1A–green fluorescent protein (GFP) and GFP–CFE1A constructs, respectively, by Topo cloning and subsequent recombination reaction as described by Liu *et al.* (2014). The binary vectors were transiently co-expressed in leaves of *Nicotiana benthamiana* with ABD2-mCherry or red fluorescent protein (RFP)–HDEL via agroinfiltration (Waadt and Kudla, 2008). The images were taken by a spinning-disc confocal microscope (UltraView VoX, Perkin Elmer) as described by Liu *et al.* (2014).

Fluorescent staining, and microscopic and quantitative analyses of the actin cytoskeleton

Fibre-bearing seeds were carefully dissected from fresh bolls obtained at 5 and 8 DPA. F-actin staining by Alexa Fluor® 488 phalloidin (Molecular Probes, Invitrogen) was performed according to the manufacturer's instructions. The seeds were washed twice with phosphate-buffered saline (PBS; pH 7.0) for 10 min each. After fixing with 4% (w/v) paraformaldehyde in PBS for 10 min, the fibres were carefully cut from the ovules and washed twice or more with PBS, and then incubated for 20–30 min in PBS containing 1 U of Alexa Fluor® 488 phalloidin, 0.1% (v/v) Triton X-100, 40 mM HEPES (pH 7.0), 1 mM $MgCl_2$, 3 mM DTT, and 0.3 mM PMSF. After briefly rinsing in PBS, the fibres were mounted onto glass

slides and examined under a Leica DMl fluorescence microscope. At image acquisition, all settings, including excitation wavelength and emission filters (488 nm/band-pass 505–530 nm for Alexa-Fluor 488), were fixed. To evaluate actin alignment, occupancy analysis, a statistical parameter, was employed to quantify actin density, according to a previously described method (Higaki *et al.*, 2010).

Accession numbers

Sequence data from this article can be found in the GenBank data libraries under accession numbers: AF072404 (*GhCFE1D*, originally named as *GhCFE1,* isolated by Yamamoto and Baird, 1998), DQ073045 (*GhCFE1A*), XP_002521431.1 (RCOM_1063000), XP_0025 31045.1 (RCOM_0517470), XP_002273372.2 (LOC100244739), CBI23474.3 (VIT_00006712001), XP_002284551.1 (LOC10026 7532), XP_002316652.1 (POPTRDRAFT_833990), XP_0023304 25.1 (POPTRDRAFT_811252), XP_002316932.1 (POPTRDRA FT_771875), NP_176321.2 (At1g61260); NP_200241.1 (At5g54300), KF018237 (*CFE1A* A1), KF018238 (*CFE1D* D5), KF018239 (*CFE1A* TM-1), KF018240 (*CFE1D* TM-1), KF018241 (*CFE1A* Hai7124), KF018242 (*CFE1D* Hai7124), KF018243 (*GhACTa*), KF018244 (*GhACTb*).

Results

CFE1 *genes in diploid and allotetraploid cotton*

A cotton fibre cDNA library was constructed in a previous study, using ovules (1 and 3 DPA) and fibres (5–25 DPA) from *G. hirsutum* accession 7235, a germplasm line with elite fibre quality (H.H. Wang *et al.*, 2010). Through random sequencing of clones from the library, a 1274 nucleotides long cDNA clone was obtained. This cDNA encodes a predicted polypeptide of 331 amino acids which shares very high homology (96% identity) with GhCFE1 (Yamamoto and Baird, 1998). Bioinformatics analysis predicted that either of

the two encoded proteins contains a signal peptide encoded by its N-terminal fragment (first 26 amino acid residues). Additionally, Southern blotting of genomic DNA from *G. hirsutum* accession TM-1 (AADD) showed that the gene cloned in this study is present as two copies in the tetraploid species (Fig. 1A), implying that the novel gene and *GhCFE1* are two homoeologous loci in allotetraploid cotton. To investigate this, *CFE1* genomic loci were cloned and sequenced from TM-1 and *G. barbadense* cv. Hai7124 (AADD), and close relatives of the progenitor species, *G. herbaceum* L. (A genome) and *G. raimondii* Ulbrich (D genome). Gene structure and sequence alignment analysis confirmed that these *CFE1* genes, with a single copy from the diploid species and two homeologues from each allotetraploid species, contained only one intron spliced at the same site (the 200th amino acid, Fig. 1B) and are classified into two groups, A genome- and D genome-like, which were designated *CFE1A* and *CFE1D*, respectively. Phylogenetic analysis of *CFE1A* and *CFE1D* homologues suggested that the gene originally cloned from the cDNA library in this study was the transcript from the A subgenome and designated *GhCFE1A*, and *GhCFE1* (*GhCFE1D*) isolated by Yamamoto and Baird (1998) was from the D subgenome in tetraploid cotton (Fig. 1C). Based on the single nucleotide polymorphisms (SNPs) of *CFE1D* sequences between the two mapping parents, TM-1 and Hai7124 (Guo *et al.*, 2007), *GhCFE1D* was mapped on chromosome D5 (chromosome 19; Fig. 1D), implying that *GhCFE1A* is present on the homeologous chromosome A5 (chromosome 5).

Motif analysis indicated that GhCFE1 homeologues in cotton contains two conserved domains of unknown function (DUFs), DUF761 (residues 293–330; Fig. 2A) and DUF4408 (residues 42–75) which is found at the N-terminus of
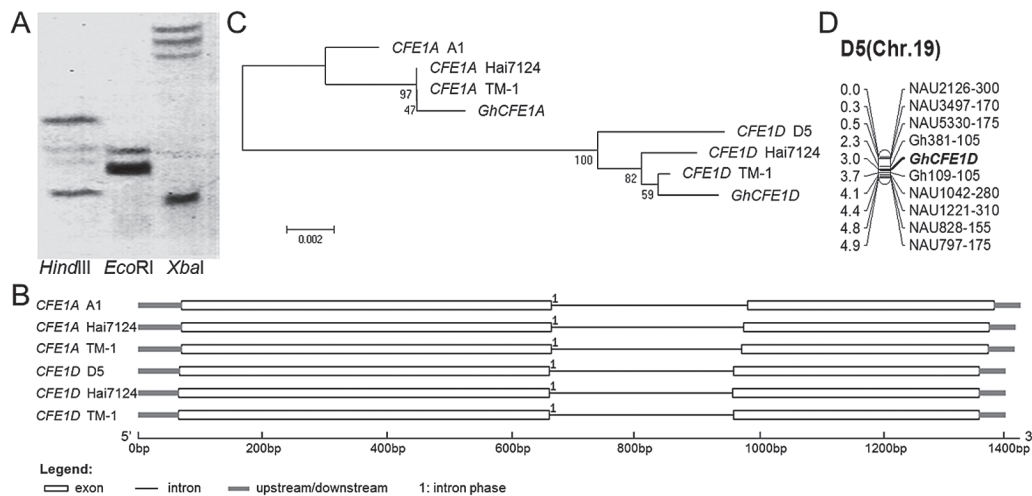

Fig. 1. Genomic organization of *CFE1* genes in diploid and allotetraploid cotton. (A) Southern blot analysis of the gene isolated in this study from a normalized cDNA library in allotetraploid cotton. Genomic DNA was digested with *Hind*III, *Eco*RI, and *Xba*I, and hybridized with a 458 bp fragment of the novel gene which contains one *Xba*I restriction site. (B) Gene structure analysis of *CFE1* genomic sequences from *G. herbaceum* (A1), *G. raimondii* (D5), *G. barbadense* (AADD, Hai7124), and *G. hirsutum* (AADD, TM-1). The exons and introns are represented by different characters. (C) Phylogenetic analysis based on nucleic acid sequences of *CFE1* genes from different cotton species. 'GhCFE1A', cDNA sequence isolated in this study; 'GhCFE1D', cDNA sequence isolated by Yamamoto and Baird (1998; originally named *GhCFE1*); '*CFE1A* A1', genomic *CFE1* sequence in *G. herbaceum* (A1); '*CFE1D* D5', genomic *CFE1* sequence in *G. raimondii* (D5); '*CFE1A* TM-1' and '*CFE1D* TM-1', A and D subgenome genomic *CFE1* sequence in *G. hirsutum* TM-1(AADD), respectively; '*CFE1A* Hai7124' and '*CFE1D* Hai7124', A and D subgenome genomic *CFE1* sequence in *G. barbadense* Hai7124 (AADD), respectively. (D) Chromosomal localization of *GhCFE1D*, the homeologous gene of *GhCFE1A*.
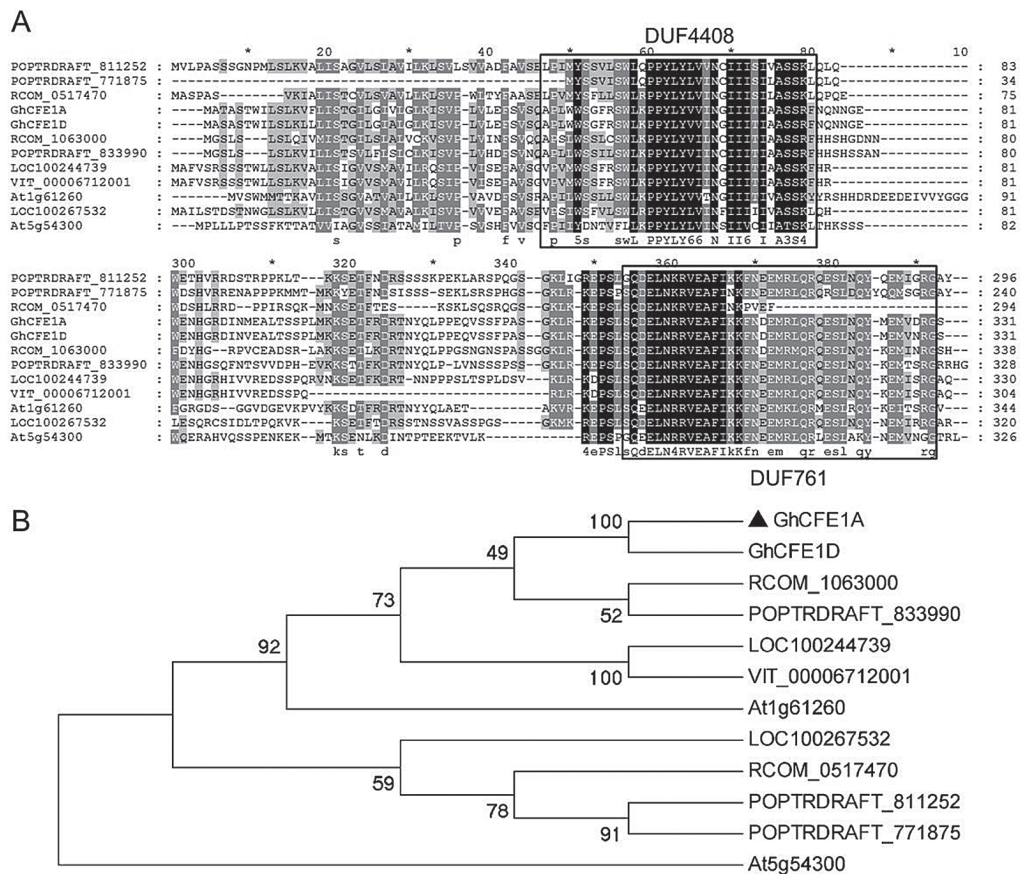
Fig. 2. Two conserved domains with unknown function (DUFs) were found among the CFE proteins from different plant sources. (A) Multiple sequence alignment analysis of GhCFE1A/D and other plant CFE proteins uncharacterized and not functionally analysed from *Ricinus communis* (RCOM_1063000 and RCOM_0517470), *Vitis vinifera* (LOC100244739, LOC100267532, and VIT_00006712001), *Populus trichocarpa* (POPTRDRAFT_833990, POPTRDRAFT_811252, and POPTRDRAFT_771875), and *Arabidopsis thaliana* (At1g61260 and At5g54300). Accession numbers are listed in the Materials and methods. (B) Phylogenetic analysis of CFE proteins. The Neighbor–Joining tree was generated from the amino acid sequences mentioned above, and the numbers next to each node give bootstrap values from 1000 replicates.

members of the DUF761 family. The GhCFE1-homologous peptides from *Ricinus communis*, *Vitis vinifera*, and other species were identified by a database search. The proteins show high homology, especially at the N- and C-terminal domains, suggesting that these regions are important for CFE function. The protein from *Ricinus* species exhibited the highest similarity to GhCFE1, with 59% amino acid sequence identity (Fig. 2B).

GhCFE1 is correlated with cotton fibre initiation and elongation

Both RT–PCR and qRT-PCR data for the *GhCFE1* expression pattern were consistent with each other. *GhCFE1* transcripts were detected in the immature ovules of TM-1 prior to the formation of fibre initials and reached the highest level in the fibre cells at 5 DPA (Fig. 3A). At the rapid elongation stage, the expression was moderate, but the transcript abundance sharply decreased from 17 to 23 DPA in fibre cells. The roots and stems showed almost no expression, and the leaves expressed *GhCFE1* at very low levels. Moreover, *GhCFE1* transcripts were dozens of times higher in 0, 3, and 5 DPA ovules of the *fl* mutants (MD17, SL1-7-1, and Xu142 fl) than in the corresponding fibre-bearing TM-1 ovules at the same

developmental stage (Fig. 3B). These results indicate that the expression of *GhCFE1* is associated with fibre initiation and elongation; moreover, the much higher transcripts of *GhCFE1* detected in fuzzless–lintless mutants of cotton suggest that its excessive expression may not be conducive to the formation of lint and fuzz fibres.

Increased GhCFE1A *expression represses cotton seed fibre and stem trichome development*

Two overexpression constructs, driven by the 35S constitutive promoter and E6 fibre-specific promoter, were generated (Supplementary Fig. S1 at *JXB* online) and introduced into cotton by *A. tumefaciens*-mediated transformation. The empty vector pBI121 was used as the control. A total of nine independent transformed lines (T_0), including four 35S-sense-*GhCFE1A* transgenic lines (named 35SSC plants) and five E6-sense-*GhCFE1A* transgenic lines (named E6SC plants) were selected. Positive transformants were confirmed by PCR detection of *NPTII*, *35S-GhCFE1A*, and *E6-GhCFE1A*, respectively (Supplementary Fig. S2A), and Southern blot analysis (Supplementary Fig. S2B). The *GhCFE1* transcript levels were significantly higher in the ovules (0 DPA) and fibres (5–15 DPA) of all the T_3 homozygous *GhCFE1A*
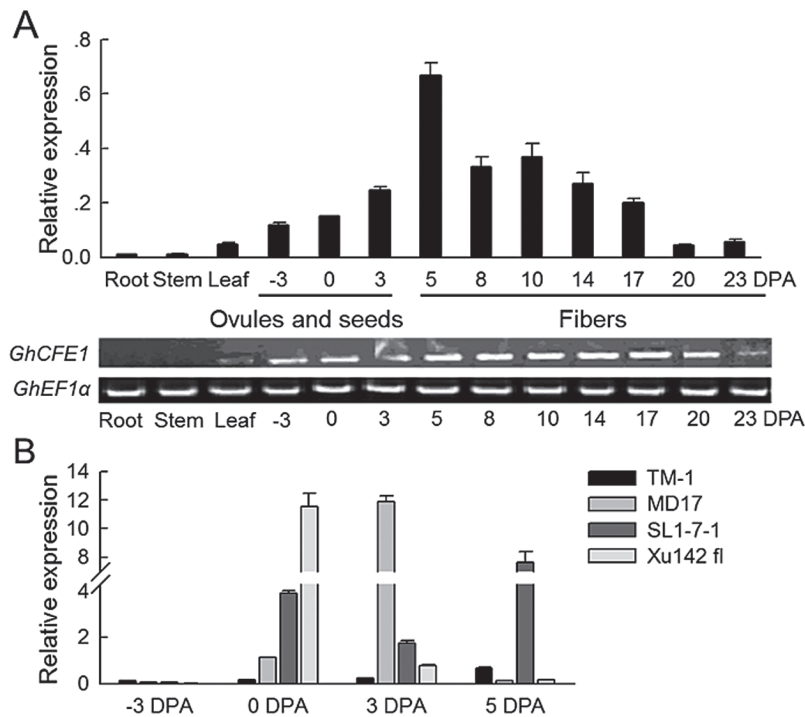
Fig. 3. Expression patterns of *GhCFE1*. (A) The expression levels of *GhCFE1* in root, stem, leaf, ovules, and seeds at –3, 0, and 3 DPA, and fibres at 5–23 DPA. Error bars represent the standard deviation of triplicate experiments, and *His3* was used as an internal control in quantitative RT-PCR (qRT-PCR). *EF1α* was used as an internal control in RT–PCR. (B) The transcripts of *GhCFE1* were highly enriched in the fuzzless–lintless mutants (MD17, SL1-7-1, and Xu142 fl) in the early stage of cotton fibre development. Error bars represent the standard deviation of triplicate experiments, and *His3* was used as an internal control.

overexpression plants, especially in the 35SSC transgenic lines, as compared with their corresponding wild-type plants (Fig. 4A; Supplementary Fig. S3). This finding is consistent with the protein levels of GhCFE1 (Fig. 4B), and the size of the GhCFE1 band was determined by heterologous expression of GhCFE1A in *E. coli* (Supplementary Fig. S4). Moreover, *GhCFE1* was sharply up-regulated in the root, stem, and leaf of the four 35SSC lines (Fig. 4A, B).

To identify further the effect of overexpression of *GhCFE1A*, the 35SSC transgenic plants of the T_3 generation were analysed for lint and fuzz development. Under electron microscopy, the initial patterns of the lint and fuzz fibre cells on the surface of the ovules at 0 DPA and the seeds at 4 DPA with the lint fibre removed to make the fuzz fibre visible, respectively, were compared between the wild-type and 35SSC line 159 (Fig. 5A). The density of both lint and fuzz fibres was significantly decreased in line 159 (153.11 ± 19.37 lint fibre and 6.42 ± 2.32 fuzz fibre initiations in an area of 250 μm×170 μm) as compared with that in the wild-type plants (219.33 ± 23.32 lint fibre and 14.45 ± 5.25 fuzz fibre initiations in an area of the same size; Fig. 5B). Similar results were found following statistical analysis of data in other lines (Supplementary Table S2 at *JXB* online). Upon detaching the mature lint fibres from seeds, the T_3 transgenic seeds were found to contain fewer fuzz fibres than wild-type and control plants (Fig. 5C). Meanwhile, the overexpression of *GhCFE1A* also altered the initial density of stem trichomes, which was obviously lower in all the 35SSC plants as compared with the wild-type plants (Fig. 5D).

Additionally, comparison of the fibre length between the *GhCFE1A*-overexpressing lines and wild-type plants revealed that lint fibre elongation was retarded in line 159 at 0 DPA (Fig. 5A) and the subsequent development stages (8–25 DPA, Fig. 6A), ultimately leading to much shorter mature fibres in 35SSC transformants than in the control plants (Fig. 6B). The survey of fibre quality in the transgenic lines and control plants showed that the transgenic line 159 had significantly shorter fibre length (24.90 ± 0.90 mm) than the wild-type (27.58 ± 0.43 mm) and control plants (27.67 ± 1.13 mm), and the fibre length of the other 35SSC transgenic lines also obviously decreased (26.47 ± 0.37, 26.28 ± 1.31, and 26.49 ± 0.59 mm for lines 155, 158, and 164, respectively, Fig. 6C). However, no significant alterations in strength and micronaire were observed in the transgenic plants. The fibre initial index and the fibre length of E6SC transgenic plants can be viewed in Supplementary Tables S2 and S3 at *JXB* online, and the level of reduction of fibre initiation and elongation is lower than in 35SSC plants. All the observations for *GhCFE1A*-overexpressing plants were associated with an increase of *GhCFE1A* transcript and protein (Figs 4–6).

Suppression of GhCFE1A exhibits no obvious phenotype alteration

A knockdown construct driven by the E6 promoter (pBI121*E6*-anti*GhCFE1A*; Supplementary Fig. S1 at *JXB* online) was introduced into cotton, and six T_0 E6-antisense-*GhCFE1A* transgenic lines (termed E6ASC plants) were
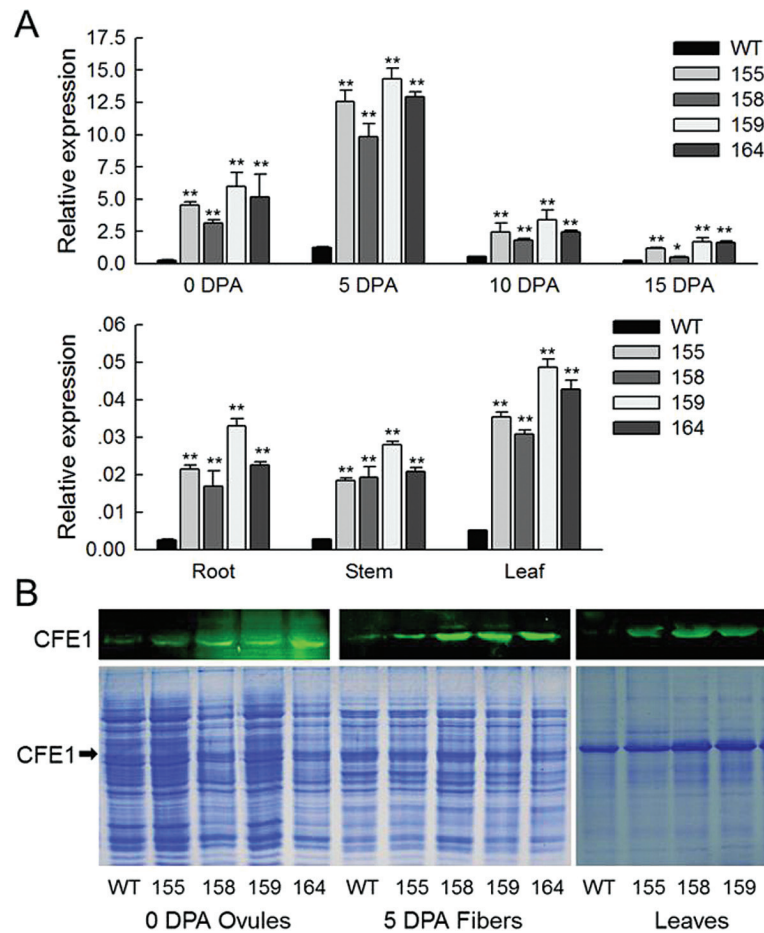
Fig. 4. The transcript and protein level analysis of GhCFE1 in wild type (WT) and *GhCFE1A*-overexpressing cotton. (A) qRT-PCR analysis of expression levels of *GhCFE1* in 0 DPA ovules, 5–15 DPA fibres, roots, stems, and leaves of WT and 35S-sense-*GhCFE1A* (35SSC) transgenic (lines 155, 158, 159, and 164) plants. Error bars represent the standard deviation of triplicate experiments, and *His3* was used as an internal control (*$P<0.05$, **$P<0.01$, by Student's *t*-test). (B) Western blotting with antibodies against GhCFE1 reveals increased abundance of GhCFE1 in 0 DPA ovules, 5 DPA fibres, and leaves of 35SSC transgenic lines (155, 158, 159, and 164). The size of the GhCFE1 band was determined by heterologous expression of *GhCFE1A* in *E. coli* (Supplementary Fig. S4 at *JXB* online), and is indicated by the black arrows. (This figure is available in colour at *JXB* online.)

obtained. Segregating T_1 plants from independent transformants were repeatedly selfed to produce T_3 seeds of transgenic homozygotes. The down-regulation of *GhCFE1* expression was confirmed by qRT-PCR (Supplementary Fig. S5A). The data showed that, especially at 10 DPA, the expression level in all the transgenic fibres was significantly reduced, which was consistent with the expression pattern of the E6 promoter (John, 1995). However, phenotypic examination showed no significant differences in the number of fuzz and lint fibre initiations at 0 and 4 DPA (Supplementary Table S2), in the density of the fuzz fibres attached to the mature seeds (Supplementary Fig. S5B), and in fibre length (Supplementary Fig. S5C) between the E6ASC and wild-type plants.

GhCFE1A decorates cortical ER, interacts with actin, and functions in fibre cell elongation

To explore how GhCFE1A protein may block the development of cotton fibre cells, GhCFE1A without the N-terminal signal peptide was fused with the GAL4-DNA-binding domain to screen a yeast two-hybrid library transformed with a cDNA library of TM-1 ovules (0 DPA) and fibres (5–20 DPA). Six candidate interaction proteins were obtained by screening 3.8×10^7 colonies (Supplementary Table S4 at *JXB* online). Of these, one clone containing a 234 amino acid long actin protein was identified. Li *et al.* (2005) previously isolated 15 *GhACT* cDNA clones from the cotton cDNA library and determined using qRT-PCR analysis that *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11* are predominantly expressed in fibre cells. However, sequence analysis revealed that *GhACT4* and *GhACT11* encoded the same putative amino acid sequence. To elucidate the interaction between the GhCFE1A and actin proteins predominantly expressed in fibre cells, the complete ORF sequences of four *GhACT* genes were cloned from TM-1 fibres, including *GhACT2*, *GhACT4*, and other two *GhACT* genes (*GhACTa* and *GhACTb*, whose encoded proteins share 98% and 99% amino acid sequence identities with GhACT1 and GhACT5, respectively) and fused them with the GAL4-activation domain. Meanwhile, new baits containing truncated GhCFE1A fragments with only one of the two DUFs, designated as ΔN (residues 76–331) and ΔC (residues 27–292, Fig. 7A) were constructed. Co-transformation of GAL4-DNA-BD–GhCFE1A and
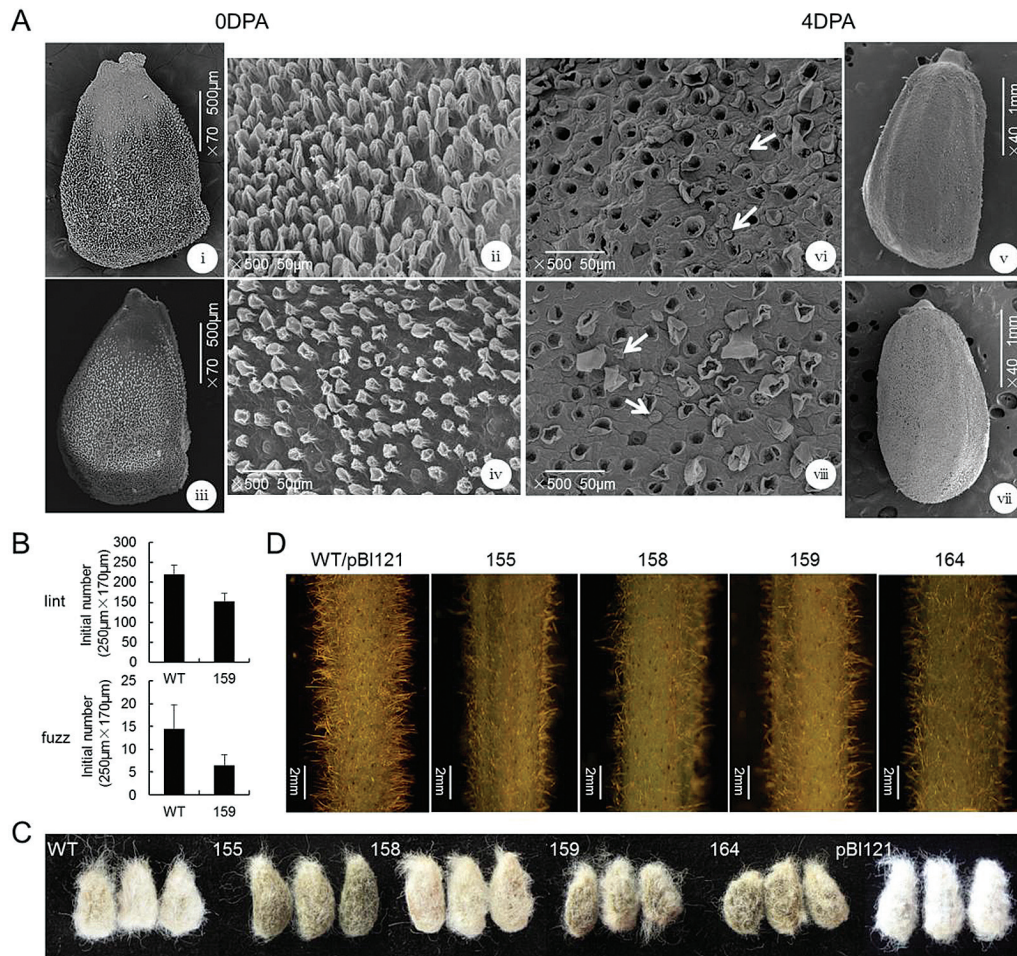
Fig. 5. Characterization and phenotype analysis of cotton seed and stem trichome initials. (A) Scanning electron microscope (SEM) images of (i–iv) 0 DPA ovules and (v–viii) 4 DPA seeds, with the lint fibre removed to make the fuzz fibre visible, illustrate the morphological differences of lint and fuzz fibre initials between the wild type (WT; i and ii; v and vi) and 35SSC line 159 (iii and iv; vii and viii). The SEM images were taken at a similar position in the middle of the ovules and seeds. White arrows indicate fuzz fibre cells (vi and viii). (B) Lint and fuzz fibre initials were counted from SEM images of ovules and seeds obtained at 0 and 4 DPA, respectively. The values were averaged over 20 images of 10 selected ovules over an area of 250 μm×170 μm for each line and the wild-type cotton. (C) Photographs of seeds from 35SSC lines, the wild type, and control (pBI121) plants with the mature lint fibres detached. (D) The stem trichomes in 35SSC transgenic cotton lines (155, 158, 159, and 164) have lower density than those in the wild-type cotton.

GAL4-AD–GhACT in yeast cells showed that GhCFE1A interacted with these four actin proteins *in vivo* (Fig. 7B); however, no interaction was detected between the truncated GhCFE1A fragments and ACTs (Fig. 7B). To investigate whether GhCFE1A and actin would be able to co-localize, constructs encoding GhCFE1A with a C- and N-terminal GFP tag, respectively, driven by the 35S promoter were constructed and transiently co-transformed into *N. benthamiana* leaf epidermal cells with ABD2-mCherry. Confocal microscopy showed that CFE1A–GFP and GFP–CFE1A not only formed filamentous structures in pavement cells which co-localized with actin bundles visualized by transiently expressing ABD2-mCherry, but also formed a polygonal tubular network morphologically similar to the ER (Fig. 7C; Supplementary Fig. S6A at *JXB* online). To confirm this localization, CFE1A–GFP and GFP–CFE1A were transiently co-expressed in *N. benthamiana* leaf epidermal cells with an ER luminal marker RFP–HDEL. The green fluorescent signal of CFE1A–GFP and GFP–CFE1A overlapped perfectly with the red fluorescent signal of RFP–HDEL, as

shown in Fig. 7D and Supplementary Fig. S6B. The co-localization pattern was further confirmed by time-lapse imaging (Supplementary Movie S1 at *JXB* online).

The ability of GhCFE1A to interact with actin was also examined *in vitro*. A co-sedimentation assay showed that GST-tagged GhCFE1A co-sedimented with actin filaments, whereas bovine serum albumin (BSA) remained in supernatants and GST–CFE1A could not sediment in the absence of actin (Fig. 7E). These results demonstrate that GhCFE1A locates on the ER network and interacts with actin both *in vivo* and *in vitro*; furthermore, the two conserved DUFs in GhCFE1A are required for the interaction.

To characterize the F-actin structure in *GhCFE1A* overexpression lines, the actin alignment in 35SSC transgenic fibres at the elongation stages (5 and 8 DPA) was determined by staining by phalloidin with green fluorescent signal and compared with that of the wild-type fibres. As shown in Fig. 8A, the actin filaments in the wild type existed longitudinally or obliquely and formed a complicated net-like structure at 5 DPA. With subsequent elongation of fibre cells, the actin
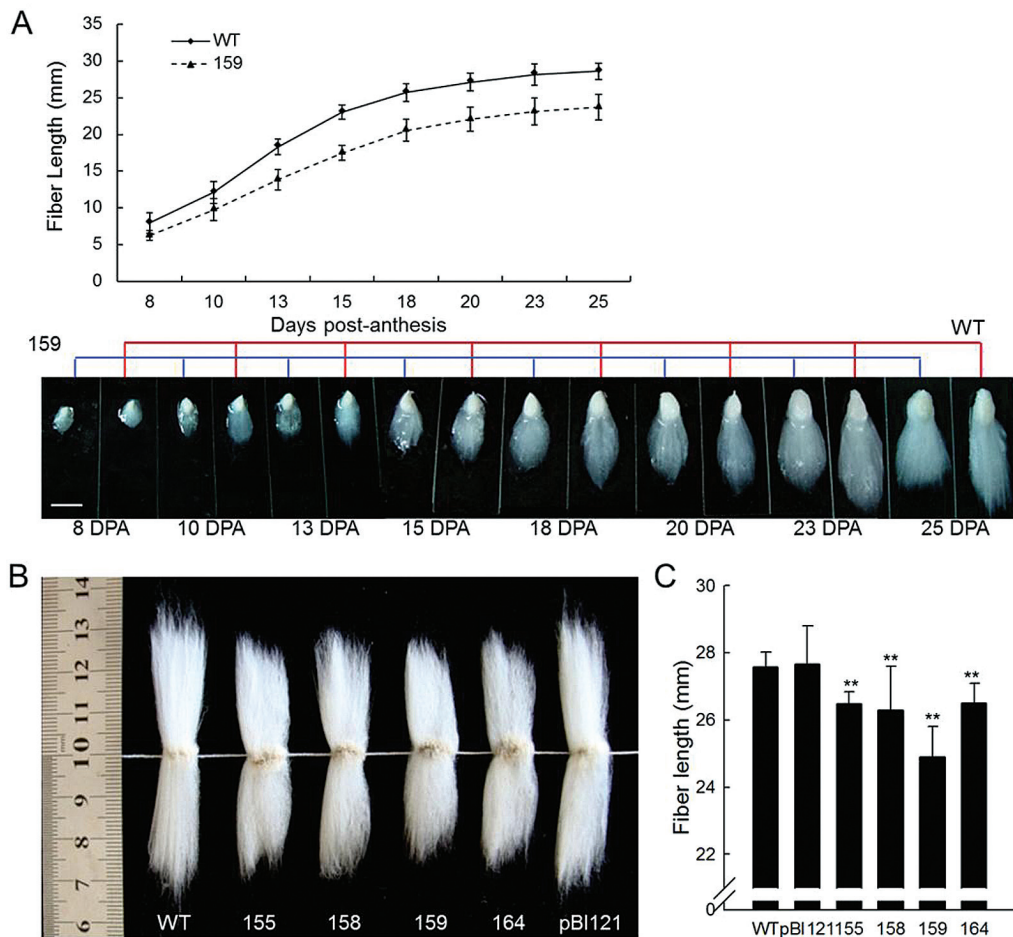
Fig. 6. Retardation of the fibre elongation process in the *GhCFE1A*-overexpressing cotton lines. (A) Dynamic curve of fibre development and phenotypes of fibre-bearing seeds in the wild type (WT) and transgenic line 159. Fibre length was quantified and the values were averaged over 10 fibre-bearing ovules of each of the three selected individual plants at every stage for the transgenic line (159) and the wild-type cotton. The stages are indicated at the bottom of the graphs. Scale bar=10 mm. (B) The mature lint fibres from 35SSC transgenic cotton lines (155, 158, 159, and 164) were shorter than those from the null control (pBI121) or wild-type fibres, particularly line 159. (C) Average fibre length of the null control, WT, and 35SSC transgenic (lines 155, 158, 159, and 164) fibres. Error bars indicate the standard deviation of triplicate experiments (**P<0.01, by Student's *t*-test). (This figure is available in colour at *JXB* online.)

cytoskeleton was comprised of relatively thick arrays or cables and displayed an increasingly complicated network. In comparison, actin filaments in the *GhCFE1A*-overexpressing fibres were obviously reduced in terms of the number of filaments, and no net-like structure were observed at 5 and 8 DPA. Changes in actin alignment were further quantified. Compared with that of wild-type fibres, the occupancy values were dramatically reduced in transgenic fibres (Fig. 8B). Thus, a decrease in actin alignment could potentially explain the short fibre phenotype in *GhCFE1A* overexpression cotton lines.

Discussion

Since the DUF761 domain proteins, GhCFE1, GhCFE2, and GhCFE3, were first identified and described as cotton fibre expressed proteins (Yamamoto and Baird, 1998), no further study has been conducted to investigate the *in vivo* function of these proteins. In the present study, it was revealed that GhCFE1A, the A genome homeologue of GhCFE1 in Upland cotton, decorated the ER network and was linked to

actin cables. Overexpressing *GhCFE1A* repressed the initiation of fibre cells, and also resulted in short fibres in the transgenic cotton lines.

GhCFE1A is associated with trichome initiation

Each cotton fibre is a single cell derived from the epidermal layer of seed coat. Seed hair development in cotton shares many similarities with leaf trichome development in *Arabidopsis* (Lee *et al.*, 2007; Guan *et al.*, 2011). However, it still remains unclear how the protodermal cells turn into fibres in cotton. In this study, it is shown that overexpressing *GhCFE1A* in cotton caused an obvious decrease in the number of lint and fuzz fibres on the surface of ovules and seeds or lower lint and fuzz indices; in addition, it resulted in a reduced number of trichomes on stems (Figs 4, 5; Supplementary Table S2 at *JXB* online). Interestingly, these findings are correlated with the up-regulated expression of *GhCFE1A* in the fuzzless–lintless mutants (MD17, SL1-7-1, and Xu142 fl) compared with linted–fuzzed TM-1 (Fig. 3B).
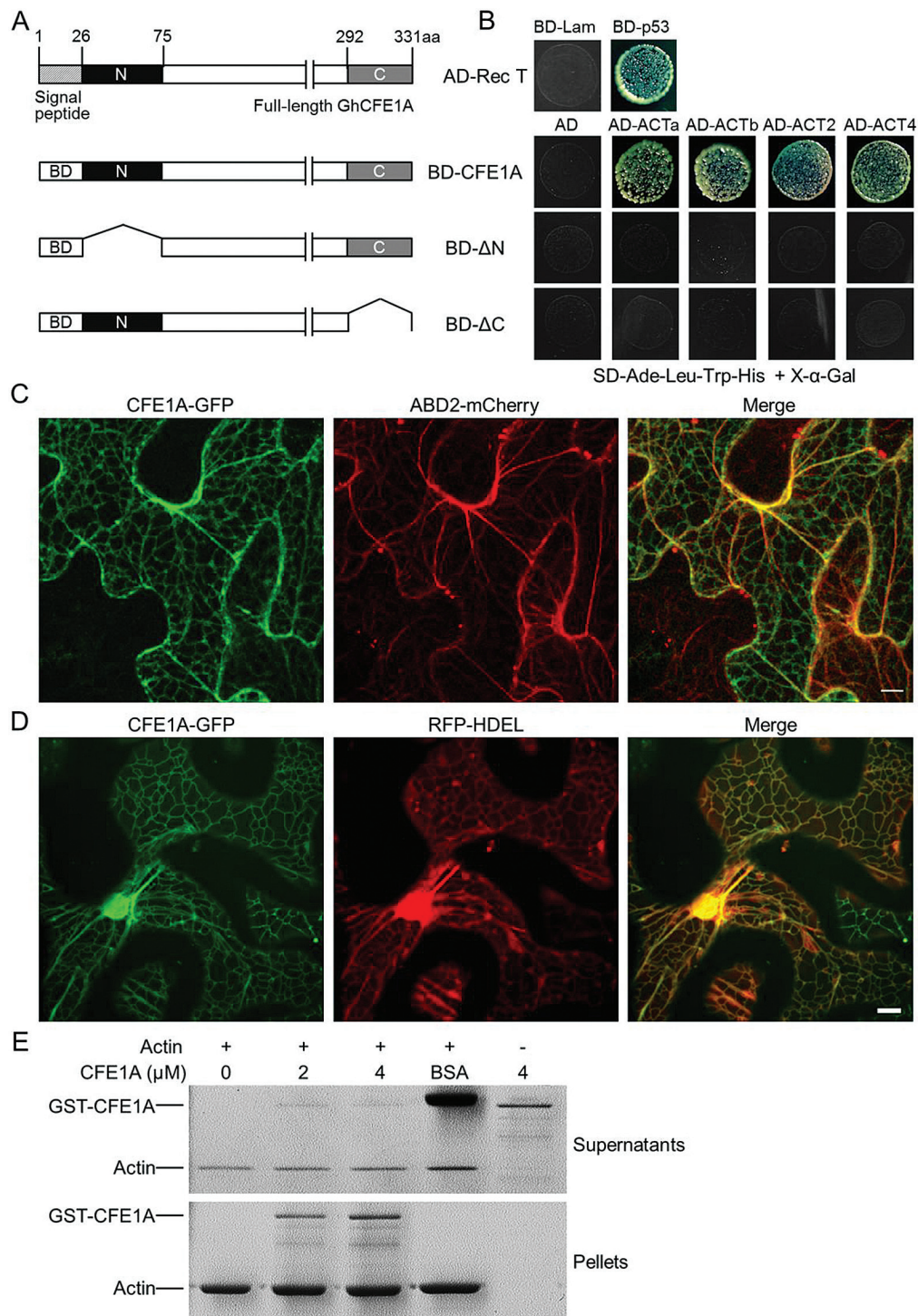
Fig. 7. GhCFE1A physically interacts with actin. (A) Structure of the GhCFE1A protein and schematic diagrams of truncated fragments fused with the GAL4-DNA-binding domain (BD) used as bait in yeast two-hybrid analysis. ΔN is the GhCFE1A protein with the N-terminal fragment deleted and ΔC is the protein without the C-terminal fragment. (B) Yeast two-hybrid assay showing that GhCFE1A interacts with GhACTa, GhACTb, GhACT2, and GhACT4. The GAL4-activation domain- (AD) fused interaction partners were retransformed into the yeast strains carrying different baits. Yeast transformants were assayed for growth on X-α-Gal synthetic complete medium without Ade, Leu, Trp, and His (SD-Ade-Leu-Trp-His). BD-p53, a fusion between the GAL4-DNA-BD and murine p53. AD-Rec T, a fusion between the GAL4-AD and large T-antigen, which interacted with p53 as the positive control. BD-Lam, a fusion of the GAL4-DNA-BD with human lamin C with AD-Rec T was used as the negative control. (C) CFE1A–GFP co-expressed with an actin filaments marker, ABD2-mCherry in tobacco (*Nicotiana benthamiana*) leaf epidermal cells. Scale bar=10 μm. (D) CFE1A–GFP co-expressed with an ER luminal marker, RFP–HDEL in tobacco leaf epidermal cells. Scale bar=10 μm. (E) Co-sedimentation assay of the actin binding activity of GhCFE1A. The presence of GST–CFE1A in the pellet indicates its co-sedimentation with F-actin.
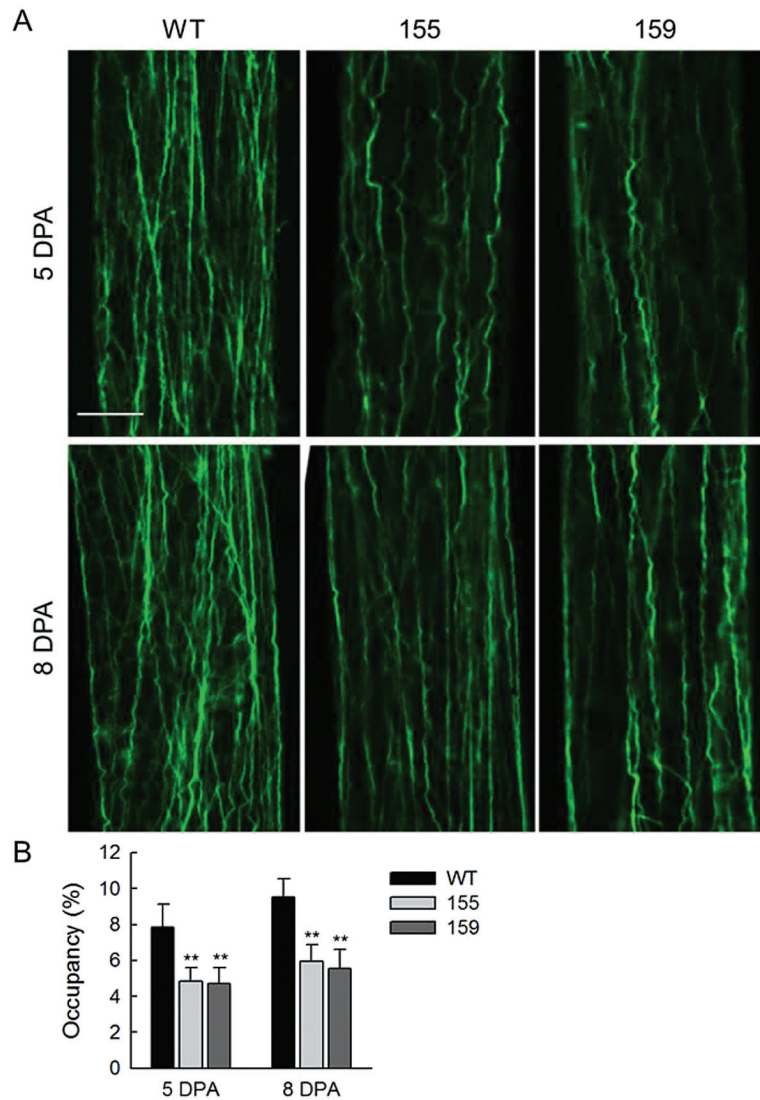
Fig. 8. F-actin organization in the wild-type and *GhCFE1A*-overexpressing fibre cells. (A) F-actin structure in five and eight DPA wild-type (WT) and *GhCFE1A*-overexpressing (lines 155 and 159) fibres. Scale bar=10 μm. (B) Values of occupancy showing the index of actin alignment in wild-type and *GhCFE1A*-overexpressing fibres. Error bars represent the standard deviation of >30 individual cells (**$P < 0.01$, by Student's *t*-test).

Therefore, the data suggested that GhCFE1A probably negatively regulates fibre cell initiation.

Previous studies have uncovered several regulators, such as GaMYB2, GhMYB25, GhMYB109, GhTTG3, and GaHOX1, which are known to regulate cotton fibre initiation and *Arabidopsis* trichome development (Wang *et al.*, 2004; Humphries *et al.*, 2005; Guan *et al.*, 2008; Pu *et al.*, 2008; Machado *et al.*, 2009). GhCFE1A may function downstream of these transcription factors; however, further studies are required to explore the molecular mechanism of *GhCFE1A* in fibre initiation.

GhCFE1A is localized to the ER and negatively regulates cotton fibre elongation through decreasing actin alignment

CFE proteins are a superfamily with unknown function, and even the homologues of this family in *Arabidopsis* are uncharacterized. GhCFE1A was shown to interact with

GhACT by yeast two-hybrid assay, which was further confirmed by *in vitro* co-sedimentation assay. However, the truncated GhCFE1A fragments with only the N- or C-terminal domain conserved had no interaction with GhACTs, implying the functional role of GhCFE1A with complete domains. Moreover, it was uncovered that GhCFE1A decorated the ER network, and was also associated with actin bundles. To the best of the authors' knowledge, this represents the first report of a protein linking the ER and actin cytoskeleton and regulating cotton fibre development.

In plant cells, the ER network is a pleomorphic, dynamic structure that pervades the entire cytoplasm (Sparkes *et al.*, 2011), and involves protein and phospholipid synthesis, their translocation, and their integration into the membrane (Voeltz *et al.*, 2002; Sparkes *et al.*, 2011). It is known that dynamic streaming of the ER network is dependent on the actin cytoskeleton in plant cells (Voeltz *et al.*, 2002; Borgese *et al.*, 2006; Sparkes *et al.*, 2009). The actin cytoskeleton has been demonstrated to participate in intracellular transport for cell

elongation (Hussey *et al.*, 2006; Staiger and Blanchoin, 2006). During cotton fibre cell elongation, the F-actin cytoskeleton is predominantly organized as thin arrays parallel to the growing axis of the fibre cells; during the elongation of fibre cells, it shows a complicated network consisting of thick and long cables and bundles (Li *et al.*, 2005; J. Wang *et al.*, 2010). Cotton fibres are highly specialized single cells which undergo enormous growth and finally increase their length 1000–3000 times the diameter of the cell (Meinert and Delmer, 1977), which would need highly active protein and phospholipid synthesis and intracellular transport. So, the ER network and actin cytoskeleton must be orchestrated to meet the requirements of synthesis and transport of the large amounts of cell wall and membrane materials.

GhCFE1A may represent an example of a protein that mediates the interplay between the ER network and the actin cytoskeleton. The *GhCFE1A* transcripts accumulated predominantly in the fibres, especially at the early stages of development and elongation, and its overexpression led to decreased cell length associated with fewer actin filaments and no formation of a net-like structure at early elongation stages. It is probable that overexpression of GhCFE1A may affect its bridging function, subsequently disorganize the actin cytoskeleton, and finally lead to impairment of cotton fibre cell elongation. Similarly, mutation of a Lumina ER protein-encoding gene, *RHD3*, also resulted in short root hairs (Schiefelbein and Somerville, 1990; Wang *et al.*, 1997; Stefano *et al.*, 2012). On the other hand, the actin motor protein, myosin XI-K, was reported to play a major role in ER network remodelling, with XI-1 and XI-2 having minor roles (Ueda *et al.*, 2010). Moreover, root hair length in class XI myosin-deficient plants was reduced ~10-fold (Peremyslov *et al.*, 2010). However, it still needs to be determined whether GhCFE1A interacts with the RHD3 or class XI myosins during cotton fibre elongation.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Construction of sense and antisense expression vectors of *GhCFE1A*.

Figure S2. Molecular confirmation of the independent transgenic lines.

Figure S3. *GhCFE1* expression in ovules and developing fibres of E6SC transgenic cotton.

Figure S4. SDS–PAGE analysis of GhCFE1A recombination protein in *Escherichia coli*.

Figure S5. Inhibition of *GhCFE1* in cotton exhibits no obvious phenotype changes.

Figure S6. GhCFE1A is located on the cortical endoplasmic reticulum (ER) and co-localizes with actin bundles.

Movie S1. Time-lapse confocal scanning microscopy of tobacco (*Nicotiana benthamiana*) leaf epidermal cells co-expressing CFE1A–GFP and RFP–HDEL.

Table S1. Oligonucleotides used in this study.

Table S2. Statistics of lint and fuzz physical parameters in 35SSC, E6SC, and E6ASC transgenic lines.

Table S3. Comparison of fibre quality parameters between transgenic lines and null control or wild-type plants.

Table S4. Clones identified by yeast two-hybrid library screening.

Acknowledgements

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