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ORIGINAL ARTICLE

An integrative analysis of four CESA isoforms specific for fiber cellulose production between *Gossypium hirsutum* and *Gossypium barbadense*

Ao Li · Tao Xia · Wen Xu · Tingting Chen · Xianliang Li · Jian Fan · Ruyi Wang · Shengqiu Feng · Yanting Wang · Bingrui Wang · Liangcai Peng

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Abstract Cotton fiber is an excellent model system of cellulose biosynthesis; however, it has not been widely studied due to the lack of information about the cellulose synthase (CESA) family of genes in cotton. In this study, we initially identified six full-length *CESA* genes designated as *Gh*CESA5–*Gh*CESA10. Phylogenetic analysis and gene co-expression profiling revealed that CESA1, CESA2, CESA7, and CESA8 were the major isoforms for secondary cell wall biosynthesis, whereas CESA3, CESA5, CESA6, CESA9, and CESA10 should involve in primary cell wall formation for cotton fiber initiation and elongation. Using integrative analysis of gene expression patterns, CESA protein levels, and cellulose biosynthesis in vivo, we detected that CESA8 could play an enhancing role for rapid and massive cellulose accumulation in *Gossypium hirsutum* and *Gossypium barbadense*. We found that *CESA2* displayed a major expression in non-fiber tissues and that *CESA1*, a housekeeping gene like, was predominantly expressed in all tissues. Further, a dynamic alteration was observed in cell wall composition and a significant discrepancy was observed between the cotton species during fiber elongation, suggesting that pectin accumulation and xyloglucan reduction might contribute to cell wall transition. In addition, we discussed that callose synthesis might be regulated in vivo for massive cellulose production during active secondary cell wall biosynthesis in cotton fibers.

Keywords Cellulose · CESA · Cotton fiber · Secondary cell wall biosynthesis

Abbreviations

CalS	Callose synthase
CESA	Cellulose synthase
CrI	Crystalline index
CSC	Cellulose synthase complex
DP	Degree of polymerization
EST	Expression sequence tag
Gb	*Gossypium barbadense*
Gh	*Gossypium hirsutum*
Kor	Korrigan
RACE	Rapid-amplification of cDNA ends
SGT	Sterol glycosyltransferase
UBQ	Ubiquitin

A. Li and T. Xia contributed equally to the work.

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A. Li · T. Xia · W. Xu · T. Chen · X. Li · J. Fan · R. Wang · S. Feng · Y. Wang · L. Peng (✉)
National Key Laboratory of Crop Genetic Improvement and National Centre of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan 430070, China
e-mail: lpeng@mail.hzau.edu.cn; liangcaipeng@gmail.com

A. Li · T. Xia · W. Xu · T. Chen · X. Li · J. Fan · R. Wang · S. Feng · Y. Wang · L. Peng
Biomass and Bioenergy Research Centre, Huazhong Agricultural University, Wuhan 430070, China

A. Li · X. Li · J. Fan · R. Wang · S. Feng · Y. Wang · B. Wang · L. Peng
College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

T. Xia · W. Xu · T. Chen · L. Peng
College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

Introduction

Cellulose is the major plant cell wall component and has wide application in textile, paper, biofuel, and chemical industries (Carroll and Somerville 2009). In higher plants, cellulose is synthesized at the plasma membrane by a very large rosette complex, which is believed to simultaneously synthesize β-1,4-glucans that form microfibrils (Kudlicka and Brown 1997; Doblin et al. 2002). Cotton (*Gossypium* spp.) is an important fiber crop worldwide. The fiber quality greatly determines its economic and commercial values (Haigler et al. 2005). Cotton fibers are single cells that originate from the epidermal layer of the ovular surface and synchronously elongate within the boll (Maltby et al. 1979; Ryser 1985). In contrast to other plant cells, cellulose constitutes more than 90 % of the dry weight of mature cotton fiber cells (Meinert and Delmer 1977). Hence, cellulose content and properties such as degree of polymerization (DP) and crystalline index (CrI) are important factors that determine cotton fiber quality (Timpa and Triplett 1993; Hu and Hsieh 1997; Xie et al. 2013; Xu et al. 2012). Given its unique property, cotton fiber has been considered as the model system for understanding the plant cellulose biosynthesis (Pear et al. 1996).

Fiber development occurs in four distinct but overlapping stages: initiation (primary cell wall formation), elongation (cell wall transition), secondary wall synthesis, and wall deposition/maturation (Basra and Malik 1984; Ruan and Chourey 1998; Lee et al. 2007). The dynamic alternation of cell wall composition, including cellulose, hemicelluloses (xyloglucan and xylan), callose, and pectin, during fiber development has not been described well for the two typical species, *G. hirsutum* (*Gh*) and *G. barbadense (Gb)* (Meinert and Delmer 1977; Hayashi and Delmer 1988; Vaughn and Turley 1999; Lee et al. 2010). Given the high fiber yield of *G. hirsutum* and the good quality of *G. barbadense* fibers, both species can be used for identifying the major genes that determine cotton fiber yield and quality (Chaudhary et al. 2008; Ghazi et al. 2009; Zhu et al. 2011). To date, numerous genes are believed to be predominantly expressed during fiber development, whereas only a few genes are specifically or preferentially expressed in a specific stage (Pear et al. 1996; Laosinchai et al. 2000; Ji et al. 2003; Ghazi et al. 2009; Pang et al. 2010).

Little progress has been made on plant cellulose biosynthesis until two cellulose synthase genes (*CESA1, 2* from original terms *CelA1, 2*) were isolated from cotton fibers (Pear et al. 1996). Both genes are highly expressed during active secondary wall synthesis in developing cotton fibers. Since then, *CESA* superfamilies have been identified in numerous higher plants (Arioli et al. 1998; Taylor et al. 1999, 2000; Fagard et al. 2000; Holland et al. 2000; Doblin et al. 2001; Burton et al. 2004; Djerbi et al. 2004, 2005; Wang et al. 2010). For instance, 10 *AtCESA* genes were reported for *Arabidopsis*. *At*CESA4, 7, and 8 are specifically required to form an essential cellulose synthase complex (CSC) typical for secondary cell wall biosynthesis (Taylor et al. 1999, 2000, 2003). Similarly, three CESA isoforms were found for secondary cell walls in rice, maize, and other plants (Tanaka et al. 2003; Appenzeller et al. 2004; Burton et al. 2004). Five CESAs have been identified in *Populus* (Song et al. 2010). Mutant analysis indicates that the silencing of each CSC member could result in plant growth defects or death (Arioli et al. 1998; Taylor et al. 1999, 2000, 2003; Fagard et al. 2000; Tanaka et al. 2003; Persson et al. 2007; Mutwil et al. 2008). Apart from CESAs, other proteins have been reported to associate with the putative CSC, such as Korrigan, sucrose synthase, COBRA-like proteins, and cytoskeleton-related proteins (Amor et al. 1995; Lane et al. 2001; Pagant et al. 2002; Roudier et al. 2005; Somerville 2006; Song et al. 2010).

Although *CESA* superfamily genes have been identified in A*rabidopsis* and other plants, only three *CESA* genes (*GhCesA1*, *2, 3*) have been reported with full sequences in cotton (Pear et al. 1996; Laosinchai et al. 2000; Kim and Triplett 2001; Grover et al. 2004). Unlike *GhCESA1, 2* showing a predominate expression specific for fiber secondary cell wall synthesis (Pear et al. 1996; Triplett and Kim 2006), *GhCESA3* displays an expression throughout fiber development (Laosinchai et al. 2000). Given the absence of available information for all *GhCESA* sequences, understanding the cellulose biosynthesis and its impact on cotton fiber property remains difficult. In this study, we initially cloned six full-length cDNAs of *GhCESA* genes (*GhCESA5, 6, 7, 8, 9, and 10*) from cotton (*Gh*) fibers, and found four isoforms (*Gh*CESA1, 2, 7, and 8) that mainly participate in secondary cell wall formation. Based on the analysis of two distinct cotton species (*Gh* and *Gb*), *Gh*CESA8 is found to act as an "enhancer" specific for substantial cellulose production in cotton fibers.

Materials and methods

Plant growth and tissue collection

Seeds of two cotton species, *Gossypium hirsutum* (Huamian 99) and *Gossypium barbadense* (Junhai 1), were provided by Guozheng Yang from Huazhong Agricultural University (HZAU) and Xianda Yi from Hubei Academy of Agricultural Science, and cotton plants were grown in HZAU experimental station. For collection of the different stage fibers, cotton bolls were tagged at initial anthesis and the corresponding bolls were harvested at 9, 14, 19, 24, 29,

34 dpa (day past anthesis). While harvested, fibers were gently and quickly removed from ovules, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

Cotton seedlings were cultured in growth chamber at 28 °C for dark and light treatments. After seed germination, the seedlings were growing under dark for 3 days as D3 samples, and then transferred to light for 3 and 6 days as L3 and L6, respectively. D3/L3/L6 hypocotyls tissues were collected and stored at −80 °C. Cotyledons were obtained from 1-week-old seedlings, and other tissues (roots/stems/leaves) were collected from 2-week-old plants.

CESA gene cloning and sequence analysis

The 3′ends of *CESA5, 6, 7, 8, 9, 10* were isolated by a rapid amplification of cDNA ends (3′RACE) technique as described by Frohman et al. (1988), and then the 5′cDNA regions were cloned by 5′RACE using the 5′-Full RACE kit (TaKaRa). Based on the both ends sequence information, full-length cDNAs of *CESA5, 6, 7, 8, 9, 10* were finally identified. The resulting protein sequences were aligned with 55 CESA proteins from *Populus trichocarpa, Hordeum vulgare, Oryza sativa, Arabidopsis thaliana, Zea mays* using ClustalX2 program (Online resource 1).

Real-time PCR

Total RNA was isolated from the collected tissues using the method described by Wu and Liu (2004). The first strand cDNA was obtained using M-MLV Reverse Transcriptase (Promega). Real-time PCR was carried out on Bio-rad MyCycler thermal cycler with the SYBER premix ExTaq (TakaRa) according to the manufacturer's instruction, and *UBQ7* was used as the internal control. All the primers used were listed in Online resource 2, and the assays were carried out in biological triplicate.

Plant cell wall fractionation

The collected plant tissues were heated at 120 °C for 20 min to inactivate samples, dried at 65 °C until constant weight, grounded using a knife-mill and passed through a 40-mesh screen. The polysaccharides were extracted as described previously (Peng et al. 2000). For pectin extraction, the crude cell wall material was suspended in 0.5 % (w/v) ammonium oxalate and heated for 1 h in a boiling water bath, and the supernatants were combined as total pectin. The remaining pellet was further suspended in 4 M KOH containing 1.0 mg mL^{-1} sodium borohydride for 1 h at 25 °C, and the combined supernatant was neutralized with acetic acid, dialysed and lyophilized as hemicelluloses. The KOH non-extractable residue was defined as crude cellulose. The experiments were performed in biological triplicate.

Colorimetric assay of hexoses, pentoses and uronic acids

UV/VIS Spectrometer (MAPADA, Shanghai) was used for assay of hexoses, pentoses and uronic acids. Hexoses were detected by the anthrone/H_2SO_4 method (Fry 1988), pentoses by the orcinol/HCl method (Dische 1962) and uronic acids by the m-hydroxybiphenyl/H_2SO_4 method (Blumenkrantz and Asboe-Hansen 1973). Total pectin was calculated subjective to the sum total of hexoses, pentoses and uronic acids, and cellulose was analyzed by accounting for hexoses. The standard curves for hexoses, pentoses and uronic acids were drawn using D-glucose, D-xylose and D-galacturonic acid, respectively. D-glucose, D-xylose, ferric chloride and orcinol were purchased from Sinopharm Chemical Reagent (Shanghai, China), anthrone and m-hydroxybiphenyl from Sigma-Aldrich, and D-galacturonic acid from Fluka Chemical Co. Because the high pentose level in the sample could affect the absorbance reading at 620 nm for hexose content by the anthrone/H_2SO_4 method, the deduction from pentoses reading at 660 nm was carried out for final calculation of hexose levels, which was verified by GC–MS analysis. All experiments were carried out in biological triplicate.

Monosaccharide determination of hemicelluloses by GC–MS

The hemicellulose monosaccharide determination was previously described by Xu et al. (2012) with minor modification. The samples were dissolved in TFA (2 M) and heated in a sealed tube at 121 °C in an autoclave (15 psi) for 1 h, and myo-inositol was added as the internal standard. The samples were then prepared for GC–MS (Shimadzu GCMS-QP2010 Plus) as described (Peng et al. 2000). GC–MS analytical conditions: Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25 μm df column. Carrier gas: He. Injection method: split, 250 °C. Injection volume 1.0 μL. The temperature program: from 170 °C (held for 12 min) to 220 °C (held for 8 min) at 3 °C min^{-1}. Ion source temperature 200 °C, ACQ Mode: SIM. The mass spectrometer was operated in the EI mode with ionization energy of 70 eV. Mass spectra were acquired with full scans based on the temperature program from 50 to 500 m z^{-1} in 0.45 s (Blakeney et al. 1983). Calibration curves of all analytes routinely yielded correlation coefficients 0.999 (Chen et al. 2003).

Enzyme digestion analysis of hemicelluloses

The hemicellulose samples were suspended in 2 mL 0.1 M acetate buffer (pH 4.5) for the following enzyme digestions: (1) Samples were added with 2 μL exo-1, 3-β-D-glucanase

(67 U mL^{-1}, Megazyme) and 2 μL endo-1,3-β-D-glucanase (20 U mL^{-1}, Megazyme), and the released glucose was accounted as callose. (2) Cellobiohydrolase (10 mg mL^{-1}, Megazyme) and endo-β-1,4-glucanase (755 U mL^{-1}, Megazyme) were added with the samples, and the released monosaccharides were totally accounted as xyloglucan. (3) Xylanase (3,750 U mL^{-1}, Megazyme) was added and the released xylose was accounted for xylan. All reactions were carried out under gentle shaking at 40 °C for 24 h and terminated at 100 °C for 10 min. After digestion, samples were added with ethanol to a final concentration at 75 % (v/v) and placed at −20 °C for 3 h. Then, samples were centrifuged at 3,000*g* for 10 min, and the supernatants were collected, dried as the released sugars and analyzed by GC–MS.

Detection of cellulose crystalline index

X-ray diffraction (XRD) method was previously described by Xie et al. (2013) with minor modification for cellulose crystallinity index (CrI) detection using D/MAX Uitima III instrument (Rigaku, Japan). The powders of cellulose samples (raw- and crude-cellulose) were laid on the glass sample holder (35 × 50 × 5 mm) and were analyzed under plateau conditions. Ni-filtered Cu Kα radiation ($\lambda = 0.154056$ nm) generated at voltage of 40 kV and current of 18 mA, and scanned at speed of 0.0197° s^{-1} from 10 to 45°. The crystallinity index (CrI) was estimated using the intensity of the 200 peaks (I200, $\theta = 22.5°$) and the intensity at the minimum between the 200 and 110 peaks (Iam, $\theta = 18.5°$) as follows: $CrI = 100 \times (I200 - Iam)/I200$. I200 represents both crystalline and amorphous materials, while Iam represents amorphous material. Standard error of the CrI method was detected at $\pm 0.05 \sim 0.15$ using five representative samples in triplicate.

Membrane protein extraction

The total membrane proteins in cotton fibers were extracted according to the method with minor modification (Peng et al. 2002). Cotton fibers were removed from ovules, ground into fine powder in liquid nitrogen, and homogenized at 4 °C in extraction buffer (50 mM Mops buffer, pH 7.5, 0.25 M sucrose containing protease inhibitors). The homogenate was centrifuged at 2,000*g* for 10 min at 4 °C, and the supernatant was filtered through two layers of gauze and centrifuged at 150,000*g* for 30 min. The pellet was resuspended in extraction buffer, dissolved with 0.05 % (m/v) digitonin and rotated at 4 °C for 30 min. The homogenate was finally centrifuged at 10,000*g* for 30 min, and the supernatant was defined as digitonin-soluble membrane proteins. Total membrane protein concentration was assayed by Bradford method (Bradford 1976).

CESA1 and CESA8 antibody preparation

The regions encoding the first hypervariable region of CESA1 and CESA8 were amplified by PCR using the following primers: CESA1, forward 5′- GGATCCGG TAGTCCATATGATGAAAACCTGTTG-3′, reverse 5′-C TCGAGAGC ATCCG GTGCCGGTTTATC-3′; CESA8, forward 5′-AAAGGATCCGTGGA GGGAGATGAAGA TG-3′, reverse 5′-AAAGTCGACGCATTTTCCAATCAT CCAT CC-3′. The amplified sequences were constructed into pGEX4T-3 vector, and the recombinant peptides were induced in *Escherichia coli BL21* by 0.5 mM isopropyl-β-D-thiogalactopyranoside. The purified peptides were injected into rabbits and antibodies were prepared by Nanjing GenScript Corporation. For a similar sensitivity, CESA1 and CESA8 antibodies were detected with dilution proportion at 1:100 and 1:1000, respectively. In addition, CESA1 and CESA8 each other were checked without the detectable crossing interaction.

SDS-PAGE and Western blotting

The digitonin-soluble membrane proteins were separated by 10 % SDS-PAGE and electrotransferred onto nitrocellulose membrane (Millipore). The membrane was blocked with TBS buffer (20 mM Tris–HCl, and 500 mM NaCl, pH 7.5) plus 5 % nonfat dry milk for 1 h. The membrane was rinsed with TTBS buffer (0.05 % Tween-20 in TBS) for three times, and incubated with primary antibody serum (*Gh*CESA1 antibody, 1:100 dilution; *Gh*CESA8 antibody, 1:1,000) for 1 h at room temperature. Followed with three times wash with TTBS, the membrane was incubated with secondary antibody (affinity-purified phosphatase labelled goat anti-rabbit IgG at a 1:5,000) for 1 h at room temperature. The membrane was finally washed three times with TTBS and one time with TBS (200 mM Tris–HCl, 150 mM NaCl, pH 7.5), and stained with 3,3′-diaminobenzidine (DAB)/NiCl for 3 min.

Results

Identification of the GhCESA superfamily in *G. hirsutum* fibers

Since GhCESA1 and GhCESA2 were first isolated from cotton fibers (Pear et al. 1996), a number of CESA families have been identified in *Arabidopsis* and other plants; however, only GhCESA1–GhCESA4 have been reported for cotton fibers. In line with the data on *Gh*CESA1 and *Gh*CESA4 reported by Grover et al. (2004), in the present study, we have confirmed two genes exhibiting almost the same protein sequence and neglected *Gh*CESA4. Based on

EST information, we cloned six new full-length *CESA* cDNAs in *G. hirsutum* fibers, designated as *Gh*CESA5–*Gh*CESA10 (Online resources 1, 3). Protein sequence analysis showed that *Gh*CESA7 and *Gh*CESA8 both lacked the motif 18, 23 and that *Gh*CESA1 lacked motif 16, 19, 22 (Fig. 1), indicating that the CESA protein structure in cotton is diverse.

According to available information on CESA sequences, an unrooted phylogenetic tree was generated among 9 *Gh*CESAs in cotton and 55 CESAs from *P. trichocarpa*, *H. vulgare*, *O. sativa*, *A. thaliana*, and *Z. mays* (Online resource 1). *Gh*CESA1, *Gh*CESA2, *Gh*CESA7, and *Gh*CESA8 could be classified as typical for secondary cell wall biosynthesis in plants (Online resource 4). *G. hirsutum* fibers displayed one more CESA (CESA7 or CESA8) than all other grassy plants but had one less compared with *P. trichocarpa*. Although *Gh*CESA7 and *Gh*CESA8 share 94.7 % identity at the amino acid sequence level, we found that each gene had a distinct upstream sequence (promoter region) (data not shown). In addition, *Gh*CESA3, *Gh*CESA5, *Gh*CESA6, *Gh*CESA9, and *Gh*CESA10 could be clustered into three other clades typical for primary cell wall biosynthesis in *Arabidopsis*, rice, and other plants.

Comparison of cotton fiber development between *G. hirsutum* and *G. barbadense*

We observed fiber development in *G. hirsutum* and *G. barbadense* species (Fig. 2). In general, both cotton species exhibit four distinct stages and the velocity of their cellulose biosynthesis can reach the maximum during active secondary cell wall formation (Lee et al. 2007). In the present study, the cellulose contents of *G. hirsutum* (24 dpa) and *G. barbadense* (29 dpa) species at stage IV were 32- and 28-fold higher, respectively, than those at stage II (14 dpa in Gh and 19 dpa in Gb; Fig. 2c, Online resource 5), confirming rapid and massive cellulose accumulation during cotton fiber development.

Obviously, *G. barbadense* species exhibited a delay in fiber initiation approximately 3–5 days after flowering at stage I (14 dpa; as primary cell wall biosynthesis) (Fig. 2a). However, at stage II (19 dpa), *G. barbadense* started to show faster fiber growth than *G. hirsutum*, particularly at stages III (24 dpa) and IV (29 dpa) as cell wall transition (as fiber elongation) and active secondary cell wall formation. As a consequence, *G. barbadense* exhibited much longer fibers compared with *G. hirsutum* at stages IV (29 dpa) and V (34 dpa; Fig. 2b). The two cotton species significantly differed in their cellulose production during cell wall transition (fiber elongation) at stage III (Fig. 2c). To elaborate, compared with that at stage II, the cellulose level at stage III increased by 9-fold in *G. barbadense* (24 dpa) but only by 2.5-fold in *G. hirsutum* (19 dpa; Online resource 5). Furthermore, cellulose biosynthesis from stage IV to V remained at similar rates in both cotton species (Fig. 2c). Finally, *G. barbadense* was of a much higher cellulose crystalline index than *G. hirsutum* during fiber thickening/wall deposition from stage IV to V (Fig. 2d). These results confirmed that fiber development in *G. barbadense* was distinct from that in *G. hirsutum*, leading to the different fiber properties in terms of fiber length, cellulose production, and crystallinity. They also indicated that a biological comparison of two distinct cotton species could provide insights into cellulose biosynthesis in cotton fibers.

CESA expression patterns in *G. hirsutum* and *G. barbadense*

The co-expression profiling of nine *CESA* genes during fiber development was analyzed in *G. hirsutum* and *G. barbadense* (Fig. 3). Based on real-time PCR analysis, we found that *CESA1*, *CESA2*, *CESA7*, and *CESA8* had extremely high levels of co-expression at stage IV with much lower transcript levels at stages I and II (Fig. 3a), supporting the phylogenetic classification of *CESA1*, *CESA2*, *CESA7*, and *CESA8* as major isoforms for secondary cell wall biosynthesis. In contrast, *CESA3*, *CESA5*, *CESA6*, *CESA9*, and *CESA10* displayed consistent but relatively low transcript levels during the entire fiber

Fig. 1 Protein structure analysis of nine *G. hirsutum* CESAs

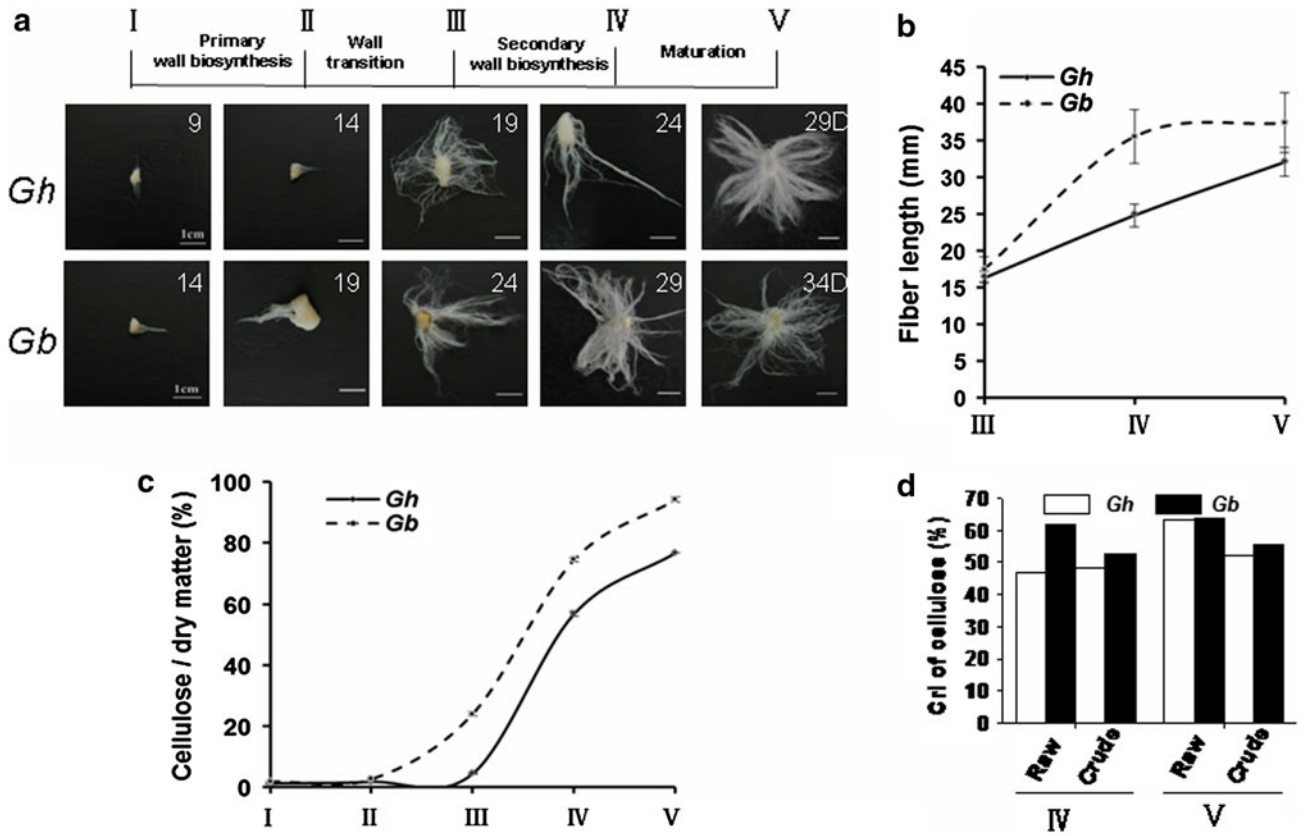

Fig. 2 Cotton fibers development in *G. hirsutum* and *G. barbadense* species. **a** Four distinct fiber development stages: numbers on the right concerns as the fiber growth days after flowering. **b** Fiber length: data given as mean ± SD ($n = 20$). **c** Cellulose content: data given as mean ± SD ($n = 3$). **d** Crystalline index (CrI) of two-type cellulose: raw cellulose obtained from the ground powder samples and crude cellulose from the pellet of 4 M KOH extraction

development in both cotton species (Online resource 6), confirming that these five genes should typically involve in primary cell wall formation.

As the *G. hirsutum* and *G. barbadense* species differed in their fiber development, we compared their *CESA1*, *CESA2*, *CESA7*, and *CESA8* expression patterns in the major stages (Fig. 3). Generally, *CESA1*, *CESA2*, *CESA7*, and *CESA8* all showed a maximum expression level at stage IV (24 dpa in Gh and 29 dpa in Gb) for active secondary cell wall biosynthesis in both cotton species (Figs. 2c, 3a). *CESA8* had the highest transcript level, whereas *CESA7* had the lowest at stage IV, suggesting that CESA8 might have a specific role in cellulose productivity in cotton fibers. A significant difference in *CESA* transcript levels was also observed at stage III: only *CESA1* had a relatively high level of expression in *G. hirsutum* (19 dpa), whereas *CESA1*, *CESA2*, and *CESA8* showed high transcript levels in *G. barbadense* (24 dpa), consistent with previously reported data about these cotton species' distinct mechanisms of cellulose production at stage III (Online resource 5).

Furthermore, we detected *CESA* gene expression in other tissues (Fig. 3b). As expected, *G. hirsutum* and *G. barbadense* exhibited high and similar *CESA1*, *CESA2*, *CESA7*, and *CESA8* expression patterns in the stem and root tissues for secondary wall biosynthesis, but they had extremely low transcript levels in the cotyledon and leaf tissues for primary cell wall formation. Compared with *CESA8*, *CESA2* displayed the highest transcript level, but *CESA7* was expressed at low levels, indicating that *CESA2* was a predominant gene for secondary cell wall formation in stem and root tissues. On the other hand, despite the minor differences between *G. hirsutum* and *G. barbadense*, *CESA3*, *CESA5*, *CESA6*, *CESA9*, and *CESA10* showed relatively consistent expression patterns in all tissues, confirming that they were typical for primary cell walls (Online resource 7).

Detection of the specific roles of *CESA1*, *CESA2*, *CESA7* and *CESA8* in hypocotyl growth

To clarify the specific role of *CESA8* in massive cellulose production, we conducted a time-course experiment on the consecutive growth of cotton seedlings under dark and light conditions (Fig. 4). Using the hypocotyl tissues collected from three distinct stages (Fig. 4a, b), the kinetics of

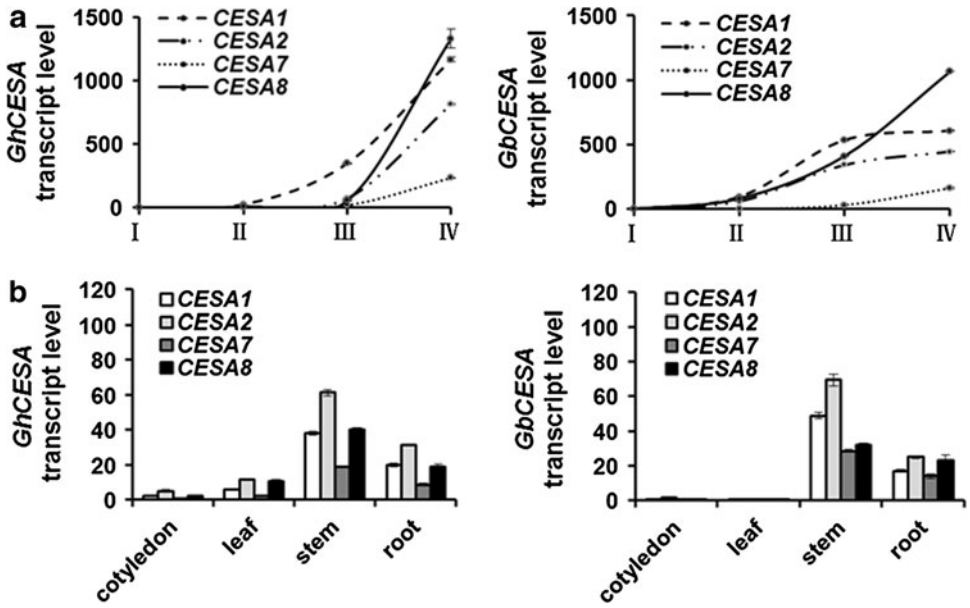

Fig. 3 *CESA1, 2, 7, 8* expression patterns by real-time PCR analysis. **a** *CESA* transcript level at cotton fibers. **b** *CESA* expression level in non-fiber tissues. *Bar* as standard error ($n = 3$)

cellulose biosynthesis from primary to secondary cell walls was analyzed, which differed between *G. hirsutum* and *G. barbadense* (Fig. 4c–e, Online resources 8, 9). Under dark conditions for 3 days, the cotton seedlings were induced for active primary cell wall biosynthesis by enhancing the expression of *CESA3*, *CESA5*, *CESA6*, *CESA9*, and *CESA10* (Online resource 9) and repressing that of *CESA1*, *CESA2*, *CESA7*, and *CESA8* (Fig. 4e). When the seedlings subjected to dark conditions for 3 days were exposed to light for 3 days, they started to be enhanced for secondary cell wall biosynthesis with increasing expression of *CESA1*, *CESA2*, *CESA7*, and *CESA8* and reduced *CESA3*, *CESA5*, *CESA6*, *CESA9*, and *CESA10* transcript levels. Under light for three more days, the seedlings remained in active secondary cell wall biosynthesis.

The *G. barbadense* species showed longer hypocotyls, higher cellulose content, and a higher cellulose crystalline index compared with *G. hirsutum* (Fig. 4), data which were in agreement with our observations on fiber development (Fig. 2). A major difference occurred between the cotton species during the cell wall transition from primary (in the dark for 3 days) to secondary (under light for 3 days) cell wall biosynthesis: during fiber development at stage III, cellulose content was increased by 2.2-fold in *G. barbadense* but only by 1.8-fold in *G. hirsutum* (Fig. 4c, Online resource 10). Despite the similar phenomena between hypocotyl growth and fiber development, we observed relatively different gene expression patterns among *CESA1*, *CESA2*, *CESA7*, and *CESA8* (Figs. 3a, 4e). For instance, *CESA2* displayed the highest expression level during hypocotyl growth, but it showed a lower transcript level during fiber development (Fig. 3a). These data indicate that *CESA8* exhibited the highest expression level during active fiber development rather than during the growth of hypocotyls and other tissues, suggesting that *CESA8* might serve as an enhancer specific for massive cellulose production in cotton fibers rather than for non-fiber tissue growth.

Assay of CESA1 and CESA8 proteins in cotton fibers

With respect to the predominant expression of *CESA8* in fiber development, we detected CESA1 and CESA8 protein levels in three major stages in the *G. hirsutum* and *G. barbadense* species by Western blot analysis (Fig. 5). With regard to fiber initiation, both cotton species at stage II were examined without clear CESA1 and CESA8 protein bands. During fiber elongation, the *G. barbadense* species at stage III (24 dpa) showed CESA1 and CESA8 protein bands, whereas *G. hirsutum* (19 dpa) remained undetectable, consistent with the findings on the much higher expression levels of *CESA1* and *CESA8* (Fig. 3a) and higher cellulose content (Fig. 2c) in *G. barbadense* than in *G. hirsutum*. Significantly, both cotton species at stage IV (24 dpa Gh; 29 dpa Gb) displayed strong CESA1 and CESA8 protein bands when the secondary cell wall biosynthesis rates reached the maximum. Using appropriate amounts of CESA1 and CESA8 antibodies at similar sensitivities to their proteins, we detected much stronger CESA8 bands than CESA1 in both cotton species, confirming our assumption about CESA8 playing an enhancing role specific for massive cellulose accumulation during active secondary cell wall biosynthesis in cotton fibers.

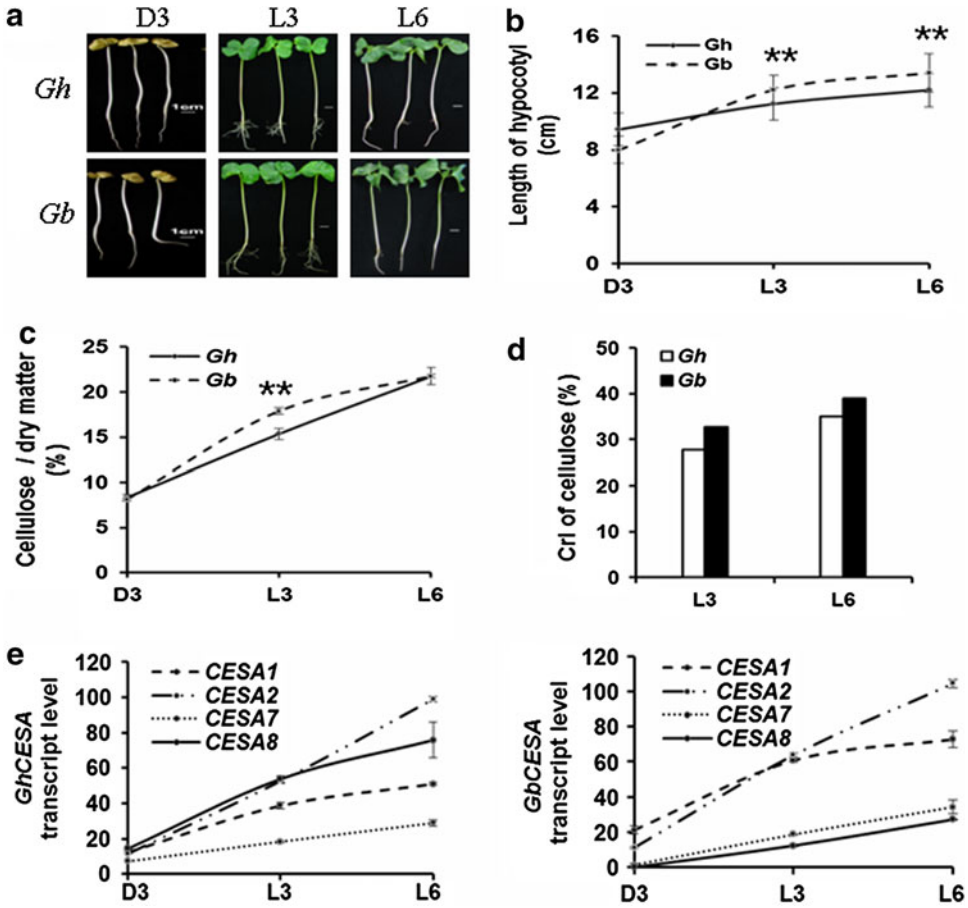

Fig. 4 Cotton seedlings growth in *G. hirsutum* and *G. barbadense*. **a** Seedlings morphology under dark and light conditions. D3, Seedlings grown under dark for 3 days; L3, D3-seedlings transferred to light for 3 days; L6, D3-seedlings under light for 6 days. **b** Hypocotyls length: *double asterisks* a significant different length between two cotton species at $P < 0.01$ ($n = 20$). **c** Cellulose content of hypocotyls, data given as mean ± SD ($n = 3$). **d** Crystalline index of crude cellulose in hypocotyls. **e** *CESA* transcript levels in hypocotyls by real-time PCR, *bar* as standard error ($n = 3$)

Characterization of cell wall dynamic alteration in cotton fibers

Due to the distinct mechanisms for fiber development between *G. hirsutum* and *G. barbadense*, a dynamic alteration in the cell wall composition of both cotton species was observed (Fig. 6). From fiber initiation (stage I) to elongation (stage III), *G. barbadense* (24 dpa) had a much higher pectin level than *G. hirsutum* (19 dpa), but the level sharply decreased in both cotton species at stage IV when the secondary cell wall biosynthesis rate reached the maximum (Fig. 6a), suggesting that pectin synthesis was active during primary cell wall formation and transition in cotton fibers. In contrast, both cotton species showed maximum callose production at stage IV, although *G. barbadense* (29 dpa) had a much lower callose level than *G. hirsutum* (24 dpa; Fig. 6b). A significant difference in hemicelluloses was found between the cotton species at stage III (Fig. 6c): *G. barbadense* (24 dpa) showed much reduction in hemicelluloses, whereas *G. hirsutum* (19 dpa) exhibited a slight increase. Furthermore, a dynamic alteration occurred in the monosaccharide composition of hemicelluloses. To elaborate, xylose from total hemicelluloses reached the highest level at stage IV (Fig. 6d), whereas galactose had a harsh drop from stage II (Fig. 6e). By comparison, the xylose from xylan increased in both cotton species at stage III (Fig. 6f).

Discussion

Cotton fiber is an excellent model system of cellulose biosynthesis and cell wall formation

Their unique properties (single cells with relatively pure cellulose) make cotton fibers an excellent model system of cellulose biosynthesis and cell wall biogenesis (Pear et al. 1996). However, the lack of information on all *Gh*CESA family genes has limited the optimal use of cotton fibers as experimental materials. In the present study, we have identified six new full-length *Gh*CESA genes and applied different experimental approaches using various samples from the fiber development phase of *G. hirsutum* and *G. barbadense*. As the different stages of fiber development present distinct cell walls, we performed *Gh*CESA gene co-expression profiling and could basically distinguish two major *Gh*CESA groups: (1) *Gh*CESA1, *Gh*CESA2, *Gh*CESA7, and *Gh*CESA8 for secondary cell wall biosynthesis and

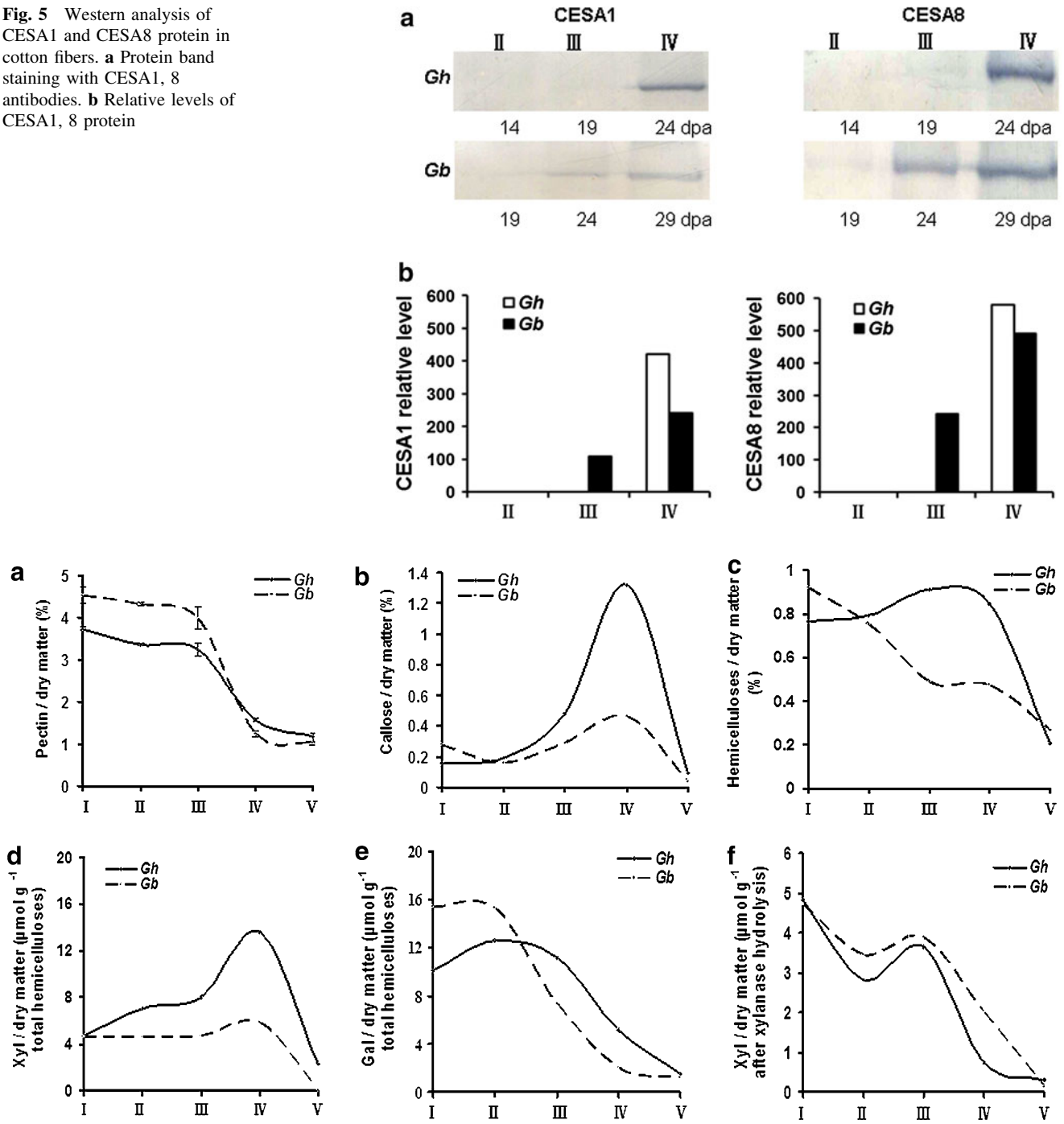

Fig. 5 Western analysis of CESA1 and CESA8 protein in cotton fibers. **a** Protein band staining with CESA1, 8 antibodies. **b** Relative levels of CESA1, 8 protein

Fig. 6 Cell wall composition in cotton fiber development. **a** Total pectin, *bar* as standard error ($n = 3$). **b** Callose. **c** Total hemicelluloses. **d** Xylose content of total hemicelluloses. **e** Galactose content of total hemicelluloses. **f** Xylose content of xylan

(2) *Gh*CESA1, *Gh*CESA3, *Gh*CESA5, *Gh*CESA6, and *Gh*CESA9 for primary cell wall formation. Furthermore, we conducted a comparative analysis of *CESA* gene-specific expressions, CESA protein contents, CESA enzyme activities, and cell wall compositions, and found that *G. hirsutum* (19 dpa) and *G. barbadense* (24 dpa) at stage III significantly differed in cell wall transition (fiber elongation). Hence, we could use the fibers from stage III (fiber elongation stage) as the optimal experimental materials with which to investigate relationships among cellulose biosynthesis, cell wall formation and fiber property in cotton and beyond.

Comparison of *G. hirsutum* and *G. barbadense* is a powerful tool for functional analysis of CESA1, CESA2, CESA7, and CESA8 in cotton

CESA mutants have been broadly used for the functional analysis of cellulose biosynthesis in *Arabidopsis* and other plants (Taylor et al. 1999, 2000, 2003; Fagard et al. 2000; Tanaka et al. 2003; Persson et al. 2007; Mutwil et al. 2008). However, partial (site mutagenesis) or complete (T-DNA insertion) silencing of each of the three CESA isoforms essential for secondary cell wall biosynthesis has resulted in similar phenotypes in plant growth defect or death (Online resource 11). Hence, silencing of *CESA1*, *CESA2*, *CESA7*, and *CESA8* in cotton would also likely lead to defective or lethal plant growth without any fiber formation or with abnormal fiber development, indicating difficulty with functional analysis of CESA in cotton fibers, particularly for the specific enhancing role of CESA8 in fiber development. Alternatively, we have demonstrated that comparing the fiber development of the two distinct cotton species *G. hirsutum* and *G. barbadense* is a powerful approach in the functional analysis of CESA1, CESA2, CESA7, and CESA8. Both cotton species showed a maximum cellulose biosynthesis rate at fiber stage IV (24 dpa Gh; 29 dpa Gb), but they displayed a significant difference at stage III (19 dpa Gh; 24 dpa Gb), which allowed us to sort out CESA8 as an enhancer for massive cellulose production in fibers. Furthermore, analysis of *CESA2*, which showed the highest transcript levels in non-fiber tissues and during the hypocotyl growth in both cotton species, not only confirmed CESA8 specification in fiber cellulose production but also indicated that CESA2 played a major role in cellulose biosynthesis in non-fiber tissues. Compared with *CESA8*, *CESA7* displayed a relatively low but very consistent expression pattern in all tissues of both cotton species, indicating that CESA7, compared with CESA8, may have functional similarities with *At*CESA7 and *Os*CESA9 in secondary cell wall formation in plants (Online resource 11). Moreover, *CESA1* presented a relatively high but consistent transcript level in all tissues of both cotton species, suggesting that CESA1 might function like a housekeeping gene in cotton.

In addition, despite some *CESA* genes have been reported with low frequent DNA sequence difference in two loci of cotton or in two cotton species (Kim et al. 2012; Lin et al. 2012), the qPCR expression levels of two cotton species were strongly consistent with their protein contents examined by Western analysis using the antibodies derived from 100 amino acids of CESA proteins. This suggested that the gene expression comparison should be useful for functional analysis of *CESA* genes, even if the same primers were designed for qPCR running in two cotton species in this study.

CESA1, CESA2, CESA7, and CESA8 are required for secondary cell wall biosynthesis in cotton

Research has shown that three CESA isoforms are required to form an essential CSC typical for secondary cell wall biosynthesis in *Arabidopsis* and other plants (Taylor et al. 1999, 2000, 2003; Tanaka et al. 2003; Appenzeller et al. 2004; Burton et al. 2004; Wang et al. 2010). However, at least five CESA isoforms have been reported to be involved in secondary cell wall formation in woody plants (Song et al. 2010). Based on phylogenetic comparisons with other plants (Online resources 1, 4) and their gene expression patterns in fiber development (Fig. 3), CESA1, CESA2, CESA7, and CESA8 were found to participate in the secondary cell wall biosynthesis in cotton fibers and other tissues. Here, we presume that the involvement of one more CESA8 may be mainly due to the requirement of massive cellulose production in fibers. This point supports our assumption that woody plants, because of their long-term substantial cellulose accumulation, need five CESA isoforms. Furthermore, although CESA7 evolved slightly later than CESA8 (Online resource 1), *CESA7* maintained a consistent and relatively low transcript level in all cotton tissues (Figs. 3, 4e), suggesting that CESA8 should be the extra isoform specific for massive cellulose production in cotton fibers.

CESA8 is an enhancer specific for massive cellulose production in cotton fibers

As described above, cellulose production in *G. hirsutum* and *G. barbadense* could be increased by 32- and 28-fold, respectively, during fiber development from primary (stage II) to secondary (stage IV) cell wall formation. We observed that CESA8 could serve like an enhancer specific for rapid massive cellulose accumulation during active secondary cell wall biosynthesis in cotton fibers. The following outcomes support this finding:

(1) *CESA8* showed the highest transcript level among the four *CESA* genes tested for maximum cellulose biosynthesis rates at stage IV in both cotton species (Fig. 3a).
(2) *CESA8* had a much lower expression level at stage III in *G. hirsutum* (19 dpa) compared with that in *G. barbadense* (24 dpa), resulting in much lower cellulose production in *G. hirsutum* (Online resource 5).
(3) *CESA8* presented a low transcript level in stem and root tissues (Fig. 3b) and during hypocotyl growth, confirming the specific role of *CESA8* in cotton fibers (Fig. 4e).
(4) CESA8 displayed a strong protein band at stage IV in both cotton species, consistent with the *CESA8* expression level (Fig. 5).

(5) The *CESA8* gene was relatively highly expressed, and the CESA8 protein was detectable with a clear band in *G. barbadense* at stage III, compared with *G. hirsutum*, resulting in a significant difference in their cellulose production (Online resource 5).

Taken together, *CESA2* gene showed the highest transcript level in all non-fiber tissues and during hypocotyl growth, thus confirming that *CESA8* was the extra gene specific for cotton fibers.

A dynamic cell wall alteration in cotton fiber property in *G. hirsutum* and *G. barbadense*

We observed a dynamic alteration in the cell wall composition of *G. hirsutum* and *G. barbadense* during fiber development, particularly at stage III. First, we found that pectin was a positive factor on fiber initiation and elongation during primary cell wall formation and transition (Figs. 2, 6). The biosynthesis of pectin precursors affected root hair elongation in *Arabidopsis* (Pang et al. 2010) and pectin controlled the extensibility and elongation of cells in ripening fruits (Goldberg et al. 1996). *G. barbadense* had also been reported to exhibit a much higher expression of the pectin methylesterase gene compared with *G. hirsutum* (Ghazi et al. 2009). These results suggest that the higher pectin accumulation in *G. barbadense*, in contrast to *G. hirsutum*, at stages I–III accounts for the relatively longer fibers that allow CESA8 to play an enhancing role in much higher cellulose production with higher crystallinity from stage III to IV (Figs. 2, 6a).

Hemicellulose type and monosaccharide composition may also affect fiber elongation. Xyloglucan is the major hemicellulose in the primary cell walls of plants, and it consists of a 1,4-β-linked glucan backbone with 1,6-α-xylosyl residues that can be substituted with galactosyl and fucosyl–galactosyl residues (Hayashi 1989). It has been reported that hemicelluloses interact with cellulose microfibrils either by becoming entrapped in the amorphous regions of the cellulose microfibrils or by forming hydrogen bonds with the microfibrils (Scheller and Ulvskov 2010; Xu et al. 2012). Xyloglucan breakdown is induced in elongating cells after exposure to auxin, resulting in the release of xyloglucan fragments (Cosgrove 2005). In the present study, we observed that *G. barbadense* started to have a considerably lower amount of xyloglucan from stage II but that *G. hirsutum* maintained a slight increase in its number (Fig. 6c), confirming that xyloglucan might negatively affect fiber elongation.

Furthermore, callose synthesis may be regulated for massive cellulose production during active secondary cell wall biosynthesis at stage IV. As plant callose synthase genes (designated as CalS) have been identified without any significant similarity with the plant CESA proteins (Cui et al. 2001; Hong et al. 2001), CESA and CalS should have independent synthase complexes but may interact for glucan synthesis. *G. barbadense* displayed threefold less callose production in vivo compared with *G. hirsutum* (Fig. 6b), suggesting that a kinetic system occurred in fiber cells in vivo for regulating cellulose and callose synthesis as well as degradation.

Finally, we observed the expression patterns of *Kor* and *SGT2*, which have been reported to associate with cellulose biosynthesis in cotton fibers (Peng et al. 2002). *G. barbadense* showed a larger increase for both genes from stage II to III, whereas *G. hirsutum* remained unchanged for *SGT2* (Online resource 12). Moreover, *G. barbadense* exhibited a much higher *SGT2* gene expression than *G. hirsutum* at stage IV, suggesting that *G. barbadense* might contain more non-CESA proteins. These data indicate that the fact that *G. barbadense* shows slightly less CESA1 and CESA8 proteins (Fig. 5) than *G. hirsutum* at stage IV may be attributed to its relatively low CESA proportion of total extracted membrane proteins. However, because *G. barbadense* started to accumulate much more cellulose than *G. hirsutum* at stage III, we determined that *G. barbadense* maintained much higher cellulose production at stages IV and V in vivo (Fig. 2).

Conclusions

*Gh*CESA gene identification revealed that CESA1, CESA2, CESA7, and CESA8 are major isoforms for secondary cell wall biosynthesis in cotton. Their expression patterns, the protein levels of CESA1 and CESA8, and cellulose biosynthesis in vivo in *G. hirsutum* and *G. barbadense* species characterize CESA8 playing an enhancing role specific for rapid and massive fiber cellulose production. With respect to the specific role of CESA8 in fiber development, a dynamic cell wall alteration occurred in both cotton species, and a significant difference between them was particularly detected during fiber elongation/cell wall transition. This study has confirmed that cotton fibers represent an excellent model system of cellulose biosynthesis and cell biogenesis in plants.

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